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Autochthon landraces characterization: proteomic and genomic approach

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Introduction

Genetic erosion in agriculture

One of the most serious consequences of human activities is the rapid extinction of many species (Frankham et al., 2004). If one species should normally disappear once in four years, in the second half of the twentieth century 1-2 species started to disappear each day (Maxim, 2010).

This alarming situation has mobilized the international scientific world which has managed to adopt the *Convention on Biodiversity* (CBD) (Hunter & Gibbs, 2007) at the so-called UN "*Earth Summit*" in Rio de Janeiro in 1992.

From the beginning of agriculture, farmers have domesticated hundreds of plant species and within them genetic variability has increased owing to migration, natural mutations and crosses, and unconscious or conscious selection (Hammer & Teklu, 2008). This gradual and continuous expansion of genetic diversity within crops went on for several millennia and its result has been a complex and continually evolving collection of local crop varieties, often referred to as landraces, that reflects interactions with wild species, adaptations to changing farming conditions, and responses to the economic and cultural factors that shape farmers priorities. The richness and range of the diversity of these landraces is now under threat because of the changing nature of agricultural production (Tripp & van der Heide, 1996). In fact, the advent of industrialized agriculture in the second half of the twentieth century led to major reductions in genetic variability in cultivated plants, called *genetic erosion*.

Genetic erosion can be defined as a permanent reduction in richness or evenness of common local alleles or the loss of combination of alleles over time in a defined area (Maxted & Guarino, 2006).

The main cause of genetic erosion is the diffusion of modern varieties from crop improvement programs (Brush, 1999). These modern varieties often provide yield increases and other advantages that result in their being sown over large areas (Tripp & van der Heide, 1996). Landraces adapted to optimal local agronomic conditions are probably the crop plant genetic resources that are most at risk of future loss through habitat destruction or by replacement by introduced elite germplasm (Brush, 1995). With the development of scientific plant breeding, high-quality and homogenous new varieties were quickly and widely distributed suppressing landraces. Yield (or yield potential), which is the characteristic of most modern varieties, is the most important criterion for the choice of a variety by a farmer (Heisey & Brennan, 1991). In fact, the farmer is less inclined to select for crop characteristics that once were important for local customs and culture (Tripp & van der Heide, 1996), but he is encouraged to grow high-

yield varieties in monoculture based on a few cultivars (Negri et al., 2009), that are often genetically similar.

Population growth, urbanization, developmental pressures on the land resources, deforestation, changes in land use patterns and natural disasters are contributing to abundant habitat fragmentation and destruction of the crops and their wild relatives (Hammer & Teklu, 2008). Overexploitation and also the introduction of invasive alien species are the other factors contributing to the loss of genetic resources. More recently, global warming and a high degree of pollution have also been recognized as further causes for the loss of biodiversity (Myers, 1994).

Last, but not least, the constant reduction in rural populations, the constant simplification of productive processes due to high manpower costs, the ageing of the maintainers, the unsuccessful farmer generation switchovers and passage of information from one generation to the next are serious threats for the maintenance of landraces (Negri, 2003).

There are several important reasons to safeguard landraces from genetic erosion.

First, local varieties are characterized by high genetic heterogenity. They have the advantage of being much better adapted to biotic and abiotic stress conditions (diseases, pests, drought, low in nutrients, etc.) (Maxim, 2010); modern varieties, instead, are characterized by genetic uniformity that leaves them vulnerable and susceptible to pathogens (Schmid, 1994) or other environmental stresses. Moreover, loss of genetic diversity reduces the ability of the plant population to genetically respond to a changing environment over time, reduces evolutionary potential, and lowers reproductive fitness (Rogers, 2004).

According to Simmonds (1991a, 1991b), the extent of genetic variation determines how well a population or species can adapt to environmental challenges such as new crop pests, diseases and drought, among others.

Second, landraces are an invaluable genetic potential for obtaining new varieties of plants and are best suited for crop cultivation in ecological systems, becoming more common (Negri et al., 2009). Also, for long term food security in the context of global warming, rich genetic diversity will be require.

In addition, these varieties may occupy a certain market niche that generates significant incomes for the rural population which can grow, thus preserving genetic diversity "on farm" (Maxim, 2010). Growing landraces can help develop local economies. Linking the local specific varieties to certain areas and localities can help strengthen the local cultural identity (Zeven, 1996).

Urgent action is needed to collect and preserve irreplaceable genetic resources (Frankel, 1974).

In situ and *ex situ* conservation are the two major strategies used in the conservation of plant genetic resources (Negri et al., 2009; Maxim, 2010). There is an fundamental difference between these two strategies: "ex situ" conservation involves the sampling, transfer and storage of a population of a certain species away from the original location where it was found, and includes the botanic gardens and storage of seed or vegetative material in gene banks (Hammer & Teklu, 2008), whereas "in situ" conservation, the conservation of diversity in its natural habitat, involves the designation, management and monitoring of the population at the location where it is currently found and within the community to which it belongs (Maxted et al., 1997). These two strategies should not be viewed as alternatives or in opposition to one another but rather should be practised as complementary approaches to conservation, each providing a safety back-up for the other. The goal of applying the two conservation strategies is ultimately to serve the present needs of plant breeders on one hand, and the need to maintain genetic resources that are always in tune with the environment to deal with future unpredictable changes on the other hand (Negri et al., 2009).

The Mediterranean region is the centre of origin of several crops and an important centre of diversification for introduced species (Hammer et al., 1992).

Italy belongs to the Mediterranean gene centre proposed by Vavilov (1927) and has a variety of different ecological, pedoclimatic, and orographic conditions. The Italian flora therefore is rich in endemics and rare plants (Pignatti, 1982) and many crops have their original domestication centre there, showing a wide genetic variability.

At the species level the loss of the existing genetic variation, proceeds relatively slowly in Italy, and most of the species mentioned under cultivation by earlier authors were still found in recent missions (Hammer et al., 1999) even in the surroundings of large cities (Hammer et al., 1996). However, there is a tremendous diversity loss at the ‘infraspecific level’. It is difficult to provide an exact estimation for this phenomenon per unit area, however, the general process can be demonstrated quite well by the decrease in the number of landraces under cultivation and by the increasing uniformity of modern agriculture (Hammer & Laghetti, 2005).

Crop genetic erosion appears to be overall more advanced in the lowlands than in the mountainous areas, and it has progressed further in the central and northern parts of Italy (Laghetti, 2009).

Hammer et al. (1996) found that there was about 73% genetic erosion of landraces in Southern Italy over a period of 30 years, and Negri (2003) found a similar loss of diversity in the

mount Amiata area (Tuscany) in only four years. Furthermore, Hammer & Laghetti (2005) examined the loss of genetic diversity in Italy using temporal comparison method. They observed a relatively high genetic erosion (13.2% p.a.) in the early years (from the 1920s to the 1950s) and estimated erosion rates between 0.48 and 4% p.a from the 1950s until the 1980s. Genetic erosion was very high in field crops such as in cereals and pulses, whereas garden crops were able to persist much longer (Hammer & Perrino, 1995).

Although it is generally accepted that significant amount of genetic erosion has occurred and is still in progress, there is little data on its amount and extent. Without remedial action, genetic erosion will inevitably increase, and the costs of replacement of diversity needed in the future by the community will be much greater (Hammer, 2004).

References

Brush, S.B. (1995) In situ conservation of landraces in centers of crop diversity. *Crop Science*, **35**, 346-354.

Brush, S.B. (1999) Genetic erosion of crop populations in centers of diversity: A revision. In: *Proceedings of the Technical Meeting on the Methodology of the FAO*, WIEWS on the PGR, Research Institute of Crop Production, Prague, Czech Republic, 34-44.

Frankel, O.H. (1974) Genetic conservation: our evolutionary responsibility. *Genetics*, **78**, 53-65.

Frankham, R., Ballou, J.D., Briscoe, D.A. (2004) *A primer of conservation genetics*, Cambridge University Press, Cambridge, UK.

Hammer, K., Knüpffer, H., Laghetti, G., Perrino, P. (1992) *Seeds from the past. A catalogue of crop germplasm in South Italy and Sicily* (ed. Germplasm Institute of C.N.R.) Bari, Italy.

Hammer, K. & Perrino, P. (1995) Plant genetic resources in South Italy and Sicily - studies towards in situ and on-farm conservation. *Plant Genetic Resources Newsletter*, **103**, 19-23.

Hammer, K., Knüpffer, H., Xhuveli, L., Perrino, P. (1996) Estimating genetic erosion in landraces - Two case studies. *Genetic Resources and Crop Evolution*, **43**, 329-336.

Hammer, K., Knüpffer, H., Laghetti, G., Perrino, P. (1999) *Seeds from the past. A catalogue of crop germplasm in Central and North Italy* (ed. Germplasm Institute of C.N.R.) Bari, Italy.

Hammer, K. (2004) Resolving the challenge posed by agrobiodiversity and plant genetic resources - an attempt. *Supplemet No. 76 to the Journal of Agriculture and Rural Development in the Tropics and Subtropics*. Kassel University Press GmbH.

Hammer, K. & Laghetti, G. (2005) Genetic erosion - examples from Italy. *Genetic Resources and Crop Evolution*, **52**, 629-634.

Hammer, K. & Teklu, Y. (2008) Plant genetic resources: Selected issues from genetic erosion to genetic engineering. *Journal of Agriculture and Rural Development in the Tropics and Subtropics*, **109**, 15-50.

Heisey, P.W. & Brennan, J. (1991) An analytical model of farmers demand for replacement of seed. *American Journal of Agricultural Economy*, 73, 44-52.

Hunter, M.L.Jr. & Gibbs, J. (2007) *Fundamentals of conservation biology*, 3rd ed., John Wiley & Sons Inc., Blackwell Publishing.

Laghetti, G. (2009) Microevolution of *Scolymus hispanicus* L. (compositae) in South Italy: from gathering of wild plants to some attempts of cultivation. In: *Agrobiodiversity and Genetic Erosion, Contributions in Honor of Prof. Dr. Karl Hammer, Supplement No. 92 to the Journal of Agriculture and Rural Development in the Tropics and Subtropics* (ed. A. Buerkert & J. Gebauer) Kassel University Press GmbH, 119-126.

Maxim, A. (2010) Conservation of genetic diversity in culture plants. *ProEnvironment*, **3**, 50-53.

Maxted, N., Ford-Lloyd, B.V., Hawkes, J.G. (1997) Complementary conservation strategies. In: *Plant Genetic Conservation: The in situ Approach* (ed. N. Maxted, B.V. Ford-Lloyd, J.G. Hawkes) Chapman & Hall, London, UK, 15-40.

Maxted, N. & Guarino, L. (2006) Genetic erosion and genetic pollution of crop wild relatives. In: *Genetic erosion and Pollution Assessment Methodologies* (ed. B.V. Ford-Lloyd, S.R. Dias, E. Bettencourt) IPGRI, Rome, Italy, 35-46.

Myers, N. (1994) Protected areas-protected from a greater 'what'? *Biodiversity and Conservation*, **3**, 411-418.

Negri, V. (2003) Landraces in central Italy: where and why they are conserved and perspectives for their on farm conservation. *Genetic Resources and Crop Evolution*, **50**, 871-885.

Negri, V., Maxted, N., Veteläinen, M. (2009) European landrace conservation: an introduction. In: *European Landraces: on farm Conservation, Management and Use*. *Biodiversity Technical Bulletin no 15*. European Cooperative Programme for Plant Genetic Resources, Rome, Italy.

Pignatti, S. (1982) *Flora d'Italia* (ed. Edagricole), Bologna.

Rogers, D.L. (2004) Genetic Erosion: No longer just an agricultural issue. *Native Plants Journal*, **5**, 112-122.

Schmid, B. (1994) Effects of genetic diversity in experimental stands of *Solidago altissima*: evidence for the potential role of pathogens as selective agents in plant populations. *Journal of Ecology*, **82**, 165-175.

Simmonds, N.W. (1991a) Selection for local adaptation in a plant breeding programme. *Theoretical and Applied Genetics*, **82**, 363-367.

Simmonds, N.W. (199lb) Genetics of horizontal resistance to diseases of crops. *Biological Reviews*, **66**, 189-241.

Tripp, R. & van der Heide, W. (1996) *The erosion of crop genetic diversity: challenges, strategies and uncertainties*. Overseas Development Institute, London.

Vavilov, N.I. (1927) Geografičeskie zakonomernosti v raspredelenii genov kul'turnych rastenii. *Trudy Prikl. Bot. Gen.*, **17**, 3.

Zeven, A.C. (1996) Results of activities to maintain landraces and other material in some European countries in situ before 1945 and what we may learn from them. *Genetic Resources and Crop Evolution*, **43**, 337-341.

Objectives

The objectives of this PhD thesis were:

1) to characterize two local lentil landraces at risk of genetic erosion cultivated in Capracotta and Conca Casale (Molise, south-central Italy) using a proteomic approach in order to identify specific landrace markers;
2) to analyze the response of Capracotta and Conca Casale lentil to salt stress at physiological and proteomic level and compare it with that of spread commercial varieties (Castelluccio di Norcia, Rascino, Colfiorito, Turca Rossa and Canadese), aiming to identify the proteins mainly involved in the response to salinity;
3) to develop an alternative molecular approach for SSR genotyping of local varieties by modifying the loop-mediated isothermal DNA amplification (LAMP) method.

Chapter I

Characterization of lentil (*Lens culinaris* Medik.) landraces from Molise.

All the studies relating with analysis of "Characterization of lentil (*Lens culinaris* Medik.) landraces from Molise" are reported in this chapter as three papers.

The first paper, published, provides the first reference proteome map of mature lentil seeds with 135 well resolved spots and showed protein spots which are essential for population discrimination, thus determining their proposition as landrace markers.

In the second paper, published, the diversity of two Molise lentil landraces, Capracotta and Conca Casale, was studied using a combination of morphological, genetic and proteomic analyses.

The third paper, in preparation, deepens the knowledge about proteomic characterization of Capracotta and Conca Casale lentil landraces, showing that the proteins which characterize the two ecotypes are mainly involved in abiotic and biotic stress responses.

INTRODUCTION

1.1 Lentil

The genus *Lens* is a member of the legume tribe *Vicieae* which includes the major legume crops such as faba bean, pea and lentil. *Lens* is a small Mediterranean genus that comprises the cultivated lentil (*Lens culinaris* Medikus subsp. *culinaris*) and 6 related taxa (Ferguson et al., 2000; Ferguson & Erskine, 2001). Scientific classification of *Lens culinaris* is given below:

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Subfamily	Fadoideae
Tribe	Vicieae
Genus	*Lens*
Species	*L. culinaris*

Cultivated lentil was divided into two varietal types, microsperma and macrosperma (Barulina, 1930), primarily based on pod and seed characters. The microsperma type has small and swollen pods and small seeds (2-6 mm diameter), red. orange or yellow cotyledons and pigmented flowers and other vegetative parts, whereas the macrosperma type was characterized by relatively large flattened pods and large seeds (6-9 mm diameter), yellow cotyledons, very light or no pigmentation on flowers and other plant parts (Figure 1).

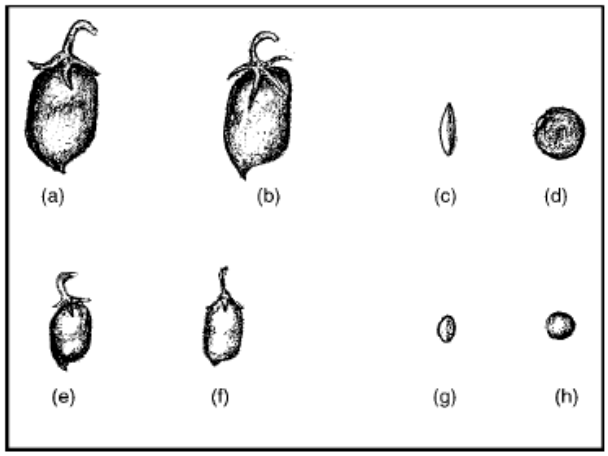

Figure 1. Variation in pods and seeds of lentil. Top row, *macrosperma* type lentil: (a and b) pods; (c) side view and (d) front view of seed. Bottom row, *microsperma* type lentil: (e and f) pods; (g) side view and (h) front view of seed (original image in Saxena, 2009).

Lentil is annual bushy herb with erect, semi-erect or spreading and compact growth habit. It has many branches with soft hairs. Its stems are slender, angular, light green in colour about 20-

30 cm in height, although there are cultivars as short as 15 cm and as tall as 75 cm (Duke, 1981; Muehlbauer et al., 1985; Saxena, 2009) (Figure 2).

Figure 2. Lentil plant structure: (a) dry seed; (b) moisture-imbibed seed; (c) a newly emerged seedling showing hypogeal germination; (d) a young seedling showing two bifoliate leaves; (e) branch with leaves, flowers and pods of *microsperma* lentil (original image in Saxena, 2009).

The rachis is 4-5 cm in length bearing 10-15 leaflets in 5-8 pairs. Generally, the upper leaves are converted into tendrils or bristle, whereas the lower leaves are mucronate (Muehlbauer et al., 1985). The leaves are alternate, compound, pinnate, usually ending in a tendril or bristly, and yellowish green, light yellow green, dull green, dark green or dark bluish green in colour. The stipules are small or absent. The axillary racemes generally bear 1-4 flowers on short peduncles having 2.5-5.0 cm length. The flowers are small, white, pink, purple, pale purple, pale blue in colour (Muehlbauer et al., 1985). The flowering proceeds acropetally. The lowermost buds open first and flowering proceeds upward and it takes about two weeks to complete opening of all the flowers on the single branch (Nezamudhin, 1970). The opening of flower occurs between 8.00 to 10.00 hrs and continues till noon and each flower remains open for about 16-24 hrs. At the end of the second day and on the third day all the opened flowers close completely and the colour of the corolla begins to fade. The setting of pods occurs after 3-4 days. The flowers have small ovaries with one or two ovules. The style is covered with a hairy inner surface. The pods are oblong, flattened or compressed, smooth with 1-2 cm in length. Pods have a curved beak, persistent calyx and contain 1-3 seeds. The seeds are biconvex, round, small, lens-shaped with 2 to 9 mm in diameter and 2 to 3 mm thick, and weigh between 2-8 g per 100 seeds (Duke, 1981). Depending on their variety and breed, however, lentil seeds can range in color from red-orange, to yellow, green, brown, or black (Sandhu & Singh, 2007). The cotyledons may be red, orange,

yellow or green, bleaching to yellow (Kay, 1979; Duke, 1981; Muehlbauer et al., 1985). The germination of seed is hypogeal.

Lentil is produced as a winter crop in the semiarid tropics, mild temperate and Mediterranean regions but is spring grown in climates with very cold winters. Lentil grows best on neutral to alkaline well-drained clay to sandy loam soils. Lentil is a quantitative long-day plant with a flowering response pattern that is independently controlled by photoperiod and temperature.

Lentil (*Lens culinaris* Medik.) is an old world legume and was probably one of the first plant species to be domesticated with wheat and barley (Bahl et al., 1993; Hancock, 2004). The oldest archaeological remains are from caves in Greece, dated 11 000 BC, and from Syria, dated 8500-7500 BC (Zohary, 1972; Hansen & Renfrew, 1978; Cubero, 1981; Zohary & Hopf, 2000). Archaeological studies showed that lentils were domesticated in the Fertile Crescent of the Near East, that coincides with the current Turkey-Syria-Iraq region (Zohary, 1972). In the 6th millennium BC, with the Neolithic revolution, this crop was common in the Nile valley, the whole Mediterranean basin, and extended to Central Europe and the Indian Subcontinent (Cubero, 1984; Zohary & Hopf, 2000; Lev-Yadun et al., 2000). From the Bronze age onward, lentils were grown wherever wheat and barley were cultivated throughout the expanding realm of Mediterranean-type agriculture (Erskine et al., 1989). Probably, lentils reached their present distribution in the Old World some 3000 years ago (Zohary & Hopf, 2000).

Due to its ancient domestication, a wide diversity within the *Lens culinaris* species was over millennia expressed in a myriad of different landraces (Solh & Erskine, 1984; Erskine, 1997). Many local types of lentil have survived because they are characterized by high adaptability to the microclimate of specific sites (Piergiovanni, 2000).

1.2 Italian landraces of lentil

Lentils have been cultivated in Italy since ancient times and until few decades ago they were one of the cheapest sources of dietary protein in rural and urban communities (Piergiovanni, 2000). Their success can be attributed to an optimal combination of climate, soil and moisture of some regions (Zucchini, 1938).

Similar to other Old World countries (Erskine, 1997; Làzaro et al., 2001), Italy's lentil cultivation is mostly based on landraces, local varieties empirically selected by farmers over time and well adapted to the agro-environment in which they have been grown for decades (Sonnante & Pignone, 2007). In the recent past, its cultivation has progressively been reduced and, consequently, many local populations were disappeared and those still being cultivated are at a high risk of genetic erosion (Ladizinsky, 1993; Piergiovanni, 2000; Negri, 2003), particularly

macrosperma types, less required by Italian market than microsperma ones (Fiocchetti et al., 2009). Several factors contributed to this decline, first of all the high production costs due to the persistence of manual cultivation practices. The main reasons for the disappearance of lentil local populations are the progressive neglect of the marginal areas, the farmers' acculturation and the switch to more remunerative crops. Unfortunately, the lack of systematic surveys on lentil diffusion in marginal areas does not allow a quantitative determination of the extent of genetic erosion (Piergiovanni, 2000). An indirect assessment may be based on the estimate of Hammer et al. (1996) who determined a level of genetic erosion higher than 60% (from 1950 to 1993) for pulse landraces of Southern Italy.

Nowadays, lentil cultivated area includes only to marginal and mountains areas of Central and Southern Italy and some small islands (Hammer et al., 1992; Hammer et al., 1999; Piergiovanni, 2000), where the survival of these remaining populations is entrusted to elderly farmers, who produce mainly for their consumption and occasionally for selling at local markets (Piergiovanni, 2000).

An overview of the current diffusion of lentils in Italy can be obtained by analysing the checklists of joint collecting missions carried out by the Germplasm Institute (IG) of the National Research Council, Bari, Italy, and the Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK), Gatersleben, Germany, from 1971 to 1998 (Table 1) (Hammer et al., 1992; Hammer et al., 1997; Hammer et al., 1999; Gladis et al., 1994; Laghetti et al., 1996; Pignone et al., 1997).

Region	Number of populations
Abruzzo	3
Basilicata	7
Calabria	2
Campania	1
Emilia-Romagna	3
Lazio	1
Marche	1
Molise	2
Apulia	12
Sardinia	8
Sicily	7
Tuscany	3
Umbria	3
Small islands	10
Total	63

Table 1. Regional distribution of Italian lentil populatios collected by IG and IPK (original image in Piergiovanni, 2000).

Lentil populations were mainly collected in marginal areas of Apennine and sub-Apennine where the process of depopulation did not take place and agriculture had maintained a rather traditional structure (Figure 3) (Piergiovanni, 2000).

Figure 3. The local lentil populatios most appreciated: (1) Onano; (2) Colfiorito; (3) Castelluccio di Norcia; (4) Valnerina; (5) S. Stefano in Sessanio; (6) Capracotta; (7) Ventotene; (8) Altamura; (9) Mormanno; (10) Ustica; (11) Villalba; (12) Pantelleria (original image in Piergiovanni, 2000).

Italian lentils are greatly appreciated by consumers for their taste and cooking qualities. Among the most known lentils, there is the Castelluccio di Norcia landrace, cultivated in the Umbria region (Central Italy), which has a good market position and potential since it is unique Italian variety to receive the PGI (Protected Geographic Identification) from the EU and from this recognition the number of hectares steadily increased, so that local communities of farmers consider it as an essential step towards the safeguarding of its survival (Torricelli et al., 2011). Other typical examples of local landraces are Colfiorito (Umbria region), Capracotta (Molise region) and S. Stefano di Sessanio (Abruzzo region) populations that are called "mountain lentils" since they are sowed in spring and harvested in summer, due to the climatic conditions of the cultivated areas, located from 1000 to 1600 m asl (Piergiovanni, 2000). Among the populations grown in hilly environments, the most interesting are Onano (Lazio region), Altamura (Puglia region) and Villalba (Sicilia region) populations, which are cultivated at an altitude of 400 to 600 m asl. Other appreciated lentils grow in the islands of Ventotene (Bozzini et al., 1988), Ustica (Hammer et al., 1999) and Pantelleria (Laghetti et al., 1996).

In recent years several studies were conducted using morphological, biochemical and DNA-based markers to characterize some Italian lentil landraces. The landraces characterization is very important to promote the survival and conservation of lentils which are under threat of extinction despite their high nutritional value and high adaptability to different environmental conditions.

Sonnante & Pignone (2001) studied a collection of lentil landraces, mostly from Italy, using RAPDs and ISSRs. First, they observed that RAPD markers detected a low level of useful polymorphic bands, whereas ISSR markers revealed a higher degree of variation, showing to be useful for distinguishing closely related genotypes. Second, the UPGMA trees, obtained using Jaccard similarity index matrices, grouped Italian populations together.

The diversity existing within and among 44 Italian populations of lentil, collected from central and southern Italy, Sardinia, Sicily and from several small islands, was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of seed storage proteins by Piergiovanni & Taranto (2005). They found that Italian lentil landraces are characterized by considerable genetic diversity and the highest variation within the electrophoretic profile of seed storage proteins was observed around 97 kDa and from 55 to 45 kDa. Only lentil from Pantelleria, Linosa and Ustica represented the exception because indistinguishable by SDS-PAGE.

Venora et al. (2006) identified five Sicilian landraces of *Lens culinaris* Medik, precisely Aragona, Bronte, Leonforte, Ustica, Villalba, and three common Canadian accessions using an image analysis system to measure seed size, shape and mean colour on individual seeds.

The eleven most known landraces (Colfiorito, Castelluccio di Norcia, Onano, S. Stefano, Capracotta, Altamura, Villalba, Ventotene, Ustica, Linosa and Pantelleria) from central and southern Italy were analysed using ISSR markers in order to assess genetic variation within and among landraces by Sonnante & Pignone (2007). The highest levels of genetic diversity were observed in some landraces from the Apennine ridge (Colfiorito, S. Stefano and Capracotta) and for one Sicilian population (Villalba); on the other hand, samples from the small Sicily islands (Linosa and Pantelleria) were less variable. Furthermore the three landraces from Pantelleria, Linosa, and Ustica showed a high level of genetic similarity showing to be very closely related to each other.

Scippa et al. (2008) investigated the genetic relationship between two landrace cultivated in Capracotta and Conca Casale (Molise, south-central Italy) and widely spread commercial varieties (Turkish, Canadian, Castelluccio di Norcia, Rascino and Colfiorito lentils) using an integrated approach consisting of studies at morphological, DNA and protein level. They reported that the two Molise lentil landraces were well differentiated from each other, and the

Conca Casale populations were separated from the commercial varieties at morphological, protein and DNA level. The Capracotta landrace, was well separated from the commercial varieties, except Castelluccio di Norcia, at DNA level showing a more complex and heterogeneous segregation at morphological and biochemical level.

Fiocchetti et al., (2009) analysed and quantified the genetic diversity within and among three macrosperma Italian lentil landraces (Onano, Altamura and Villalba), using fluorescent AFLP markers. This study showed that about 78% of the observed total genetic variation can be attributed to within population differences and around 22% is due to differences among populations, suggesting narrow genetic base among the analysed populations, confirming the tendency of Italian lentil landraces to group together.

The Santo Stefano di Sessanio lentil landraces from Abruzzo (central Italy) was characterized using three methods: morpho-agronomic traits, AFLP markers and image analysis of seeds (Torricelli et al., 2011). The authors observed that populations grown in Santo Stefano di Sessanio and in the neighbouring area basically shared most of their characteristics. However, some of the accessions bought from the local market were shown to be different from those collected from farmers.

Zaccardelli et al. (2011) investigated genetic diversity and relationships among ten lentil landraces collected in Southern and central Italy (San Gerardo, Linosa, Valle di Nevola, Altamura, Villalba, Miccula, Castelluccio di Norcia, Colfiorito, Mormanno and Colliano) , two improved lines (L 13VT and L 16VT) and two cultivars (Gaia and Itaca) using a multidisciplinary approach consisting of studies based on seed storage protein patterns and microsatellite (SSR) molecular markers. The results obtained showed that lentil landraces were grouped in different clusters and sub-clusters principally on the basis of their geographical origin and Castelluccio di Norcia, Colliano and Villalba lentils had the highest levels of genetic diversity.

References

Bahl, P.N., Lal, S., Sharma, B.M. (1993) An overview of the production and problems of Lentil in South Asia. In: *Lentil in South Asia* (ed. W. Erskine & M.C. Saxena) Proceedings of the seminar on lentils in south Asia, ICARDA, Aleppo, Syria, 1-10.

Barulina, H. (1930) Lentils of the U.S.S.R. and of other countries. *Bulletin of Applied Botany, Genetics and Plant Breeding*, **40**, 265-304.

Bozzini, A., Iannelli, P., Rossi, L. (1988) Valutazione di germoplasma di lenticchia di recente introduzione. *Informatore Agrario*, **44**, 39-45.

Cubero, J.I. (1981) Origin, taxonomy and domestication. In: *Lentils* (ed. C. Webb & G. Hawtin) Commonwealth Agricultural Bureaux, Farnham, UK, 15-38.

Cubero, J.I. (1984) Taxonomy, distribution and evolution of the lentil and its wild relatives. In: *Genetic Resources and their Exploitation-Chickpeas, Faba beans and Lentils* (ed. J.R. Witcombe & W. Erskine) M. Nijhoff & W. Junk, Boston, MA, USA, 187-203.

Duke, J.A. (1981) *Handbook of legumes of world economic importance*, Plenum Press, NewYork, USA.

Erskine, W., Adham, Y., Holly, L. (1989) Geographic distribution of variation in quantitative characters in a world lentil collection. *Euphytica*, **43**, 97-103.

Erskine, W. (1997) Lessons for breeders from landraces of lentil. *Euphytica*, **93**, 107-112.

Ferguson, M.E., Maxted, N., Van Slageren, M., Robertson, L.D. (2000) A reassessment of the taxonomy of *Lens* Mill (Leguminosae, Papilionoideae, Vicieae). *Botanical Journal of the Linnean Society*, **133**, 41-59.

Ferguson, M.E. & Erskine, W. (2001) Lentils (*Lens* L.). In: *Plant Genetic Resources of Legumes in the Mediterranean* (ed. N. Maxted & S.J. Bennett) Kluwer Academic Publishers, Dordrecht, The Netherlands, 132-157.

Fiocchetti, F., Laddomada, B., Roselli, M., Crino, P., Lucretti, S. (2009) Fingerprinting of three typical macrosperma Italian lentil (*Lens culinaris* Medik.) landraces using fluorescence-based AFLP markers. *Scientia Horticulturae*, **121**, 383-387.

Gladis, T., Hammer, K., Laghetti, G., Delogu, G.F., Perrino, P. (1994) Collecting in Sardinia, 1993. *Plant Genetic Resources Newsletter*, **98**, 18-20.

Hammer, K., Knupffer, H., Laghetti, G., Perrino, P. (1992) Seeds from the past. *A Catalogue of Crop Germplasm is South-Italy and Sicily*, Germplasm Institute-CNR, Bari, Italy.

Hammer, K., Knupffer, H., Xhuveli, L., Perrino, P. (1996) Estimating genetic erosion in landraces-two case studies. *Genetic Resources and Crop Evolution*, **43**, 329-336.

Hammer, K., Laghetti, G., Perrino, P. (1997) Proposal to make the island of Linosa/Italy as a centre for on-farm conservation of plant genetic resources. *Genetic Resources and Crop Evolution*, **44**, 127-135.

Hammer, K., Laghetti, G., Perrino, P. (1999) A checklist of the cultivated plants of Ustica (Italy). *Genetic Resources and Crop Evolution*, **46**, 95-106.

Hancock, J.F. (2004) *Plant Evolution and the Origin of Crop Species*, 2nd ed., CABI, Wallingford, UK.

Hansen, J. & J.M. Renfrew (1978) Paleolithic-Neolithic seed remains at Franchthi cave, Greece. *Nature*, **71**, 349-352.

Kay, D. (1979) Food legumes. Tropical Development and Research Institute (TPI). *TPI Crop and Product Digest*, **3**, 48-71. Tropical products Institute, London, UK.

Ladizinsky, G. (1993) Wild lentils. *Critical Reviews in Plant Sciences*, **12**, 169-184.

Laghetti, G., Hammer, K., Perrino, P. (1996) Plant genetic resources in Pantelleria and Pelagie archipelagos, Italy: collecting and conservation of local crop germplasm. *Plant Genetic Resources Newsletter*, **108**, 17-25.

Làzaro, A., Ruiz, M., de la Rosa, L., Martìn, I. (2001) Relationships between agro/morphological characters and climatic parameters in Spanish landraces of lentil (*Lens culinaris* Medik). *Genetic Resources and Crop Evolution*, **48**, 239-249.

Lev-Yadun , S., Gopher, A., Abbo, S. (2000) The cradle of agriculture. *Science*, **288**, 1602-1603.

Muehlbauer, F.J., Cubero, J.I., Summerfield, R.J. (1985) Lentil (*Lens culinaris* Medik.). In: *Grain Legume Crops* (ed. R.J. Summerfield & E.H. Roberts). Collins, London, UK, 266-311.

Negri, V. (2003) Landraces in central Italy: where and why they are conserved and perspectives for their on-farm conservation. *Genetic Resources and Crop Evolution*, **50**, 871-885.

Nezamudhin, S. (1970) Miscellanceous, Masour. In: *Pulse Crops of India* (ed. P. Kachroo) Indian Council of Agricultural Research, Krishi Bhawan, New Delhi, India, 306-313.

Piergiovanni, A.R. (2000) The evolution of lentil (*Lens culinaris* Medik.) cultivation in Italy and its effects on the survival of autochthonous populations. *Genetic Resources and Crop Evolution*, **47**, 305-314.

Piergiovanni, A.R. & Taranto, G. (2005) Assessment of genetic variation in Italian lentil populations by electrophoresis (SDS-PAGE) of seed storage proteins. *Plant Genetic Resources Newsletter*, **141**, 33-38.

Pignone, D., Hammer, K., Gladis, T., Perrino, P. (1997) Collecting in southern Sardinia (Italy), 1995. *Plant Genetic Resources Newsletter*, **109**, 7-10.

Sandhu, J.S. & Singh, S. (2007) History and origin. In: *Lentil: an Ancient Crop for Modern Times* (ed. S.S. Yadav, D.L. McNeil, P.C. Stevenson) Springer, Dordecht, The Netherlands, 1-9.

Saxena, M.C. (2009) Plant morphology, anatomy and growth habit. In: *The lentil: Botany, Production and Uses* (ed. W. Erskine, F.J. Muehlbauer, A. Sarker, B. Sharma) CABI, Wallingford, UK, 34-46.

Scippa, G.S., Trupiano, D., Rocco, M., Viscosi, V., Di Michele, M., D'Andrea, A., Chiatante, D. (2008) An integrated approach to the characterization of two autochthonous lentil (*Lens culinaris*) landraces of Molise (south-central Italy). *Heredity*, **101**, 136-144.

Solh, M. & Erskine, W. (1984) Genetic resources of lentils. In: *Genetic Resources and their Exploitation-Chickpeas, Faba beans and Lentils* (ed. J.R. Witcombe & W. Erskine) M. Nijhoff & W. Junk, Boston, MA, USA, 205-221.

Sonnante, G. & Pignone, D. (2001) Assessment of genetic variation in a collection of lentil using molecular tools. *Euphytica*, **120**, 301-307.

Sonnante, G. & Pignone, D. (2007) The major Italian landraces of lentil (*Lens culinaris* Medik.): their molecular diversity and possible origin. *Genetic Resources and Crop Evolution*, **54**, 1023-1031.

Torricelli, R., Silveri, D.D., Ferradini, N., Venora, G., Veronesi, F., Russi, L. (2011) Characterization of the lentil landrace Santo Stefano di Sessanio from Abruzzo, Italy. *Genetic Resources and Crop Evolution*, **59**, 261-276.

Venora, G., Grillo, O., Shahin, M.A., Symons, S.J. (2007) Identification of Sicilian landraces and Canadian cultivars of lentil using an image analysis system. *Food Research International*, **40**, 161-166.

Zaccardelli, M., Lupo, F., Piergiovanni, A.R., Laghetti, G., Sonnante, G., Daminati, M.G., Sparvoli, F., Lioi, L. (2011) Characterization of Italian lentil (*Lens culinaris* Medik.) germplasm by agronomic traits, biochemical and molecular markers, *Genetic Resources and Crop Evolution*.

Zohary, D. (1972) The wild progenitor and place of origin of the cultivated lentil *Lens culinaris*. *Economic Botany*, **26**, 326-332.

Zohary, D. & Hopf, M. (2000) *Domestication of Plants in the Old World*, 3rd ed., Clarendon Press, Oxford, UK.

Zucchini, M. (1938) Leguminose da granella. Note di tecnica colturale e concimazione. Comitato Nazionale per l'incremento delle concimazioni, Roma, Italy, 17-28.

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The proteome of lentil (*Lens culinaris* Medik.) seeds: Discriminating between landraces

Lentil (*Lens culinaris* Medik.) is one of the most ancient crops of the Mediterranean region used for human nutrition; an extensive differentiation of *L. culinaris* over millennia has resulted in a number of different landraces. As a consequence of environmental and socio-economic issues, the disappearance of many of them occurred in more recent times. To investigate the potential of proteomics as a tool in phylogenetic studies, testing the possibility to identify specific markers of different plant landraces, 2-D gel electrophoretic maps of mature seeds were obtained from seven lentil populations belonging to a local ecotype (Capracotta) and five commercial varieties (Turca Rossa, Canadese, Castelluccio di Norcia, Rascino and Colfiorito). 2-DE analysis resolved hundreds of protein species in each lentil sample, among which only 122 were further identified by MALDI-TOF PMF and/or nanoLC-ESI-LIT-MS/MS, probably as a result of the poor information available on *L. culinaris* genome. A comparison of these maps revealed that 103 protein spots were differentially expressed within and between populations. The multivariate statistical analyses carried out on these variably expressed spots showed that 24 protein species were essential for population discrimination, thus determining their proposition as landrace markers. Besides providing the first reference map of mature lentil seeds, our data confirm previous studies based on morphological/genetic observations and further support the valuable use of proteomic techniques as phylogenetic tool in plant studies.

Keywords:
2-DE reference map / Landrace markers / *Lens culinaris* / Seed / Storage proteins.

1 Introduction

Lentil belongs to the genus *Lens*of the Viceae tribe in the Leguminosae (Fabaceae) family, commonly known as the legume family. *Lens culinaris* Medik. originated in the Near East and was first domesticated in the Fertile Crescent around 700 BC [1]. Nowadays, lentil is an important pulse crop used for human nutrition, grown widely throughout Southern Europe, Northern Africa and Middle East regions [2, 3]. Over the time, local constraints have produced wide diversity within the *L. culinaris* species, resulting in a number of different landraces [3, 4]. In Western countries, the intense socio-economical transformations focussed the agriculture on more remunerative crops, inducing a progressive disappearance of local landraces and exposing the ones still in cultivation to a high risk of severe genetic erosion [5, 6]. Contrarily to commercial cultivars selected for their performance in specific environmental conditions, local landraces are characterized by a wide genetic variability and higher adaptation to different environmental conditions. They have evolved specific adaptive genetic traits conserved by genetic linkage, natural or human selection, which are detectable in genetic variability studies. Along the Apennine ridge of Central Italy, several mountain lentil populations are nowadays disappearing [6]. Among that Capracotta landraces have been widely appreciated for their low-input agrotechnique, favorable taste, short cooking time and low degradation during storage. The germoplasm of Capracotta lentil landraces has been characterized at morphological and genetic levels to verify its autochthonous origin [4] and has been stored at the Molise Germoplasm Bank (University of Molise, Italy).

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Abbreviation: PCA, principal component analysis

In the post-genomic era, proteomics promises to analyze dynamic molecular changes occurring during the life of an organism, not predictable at genomic level [7]. Among the most commonly used methods in proteomics, 2-DE easily allows resolution and visualization of thousands of protein species on a single gel, thus detecting isomorphisms, polymorphisms and structural changes, such as PTMs, which can be induced by specific ecological situations [8, 9]. Gene products, visualized as precise protein spots, are *de facto* genetic and physiological markers [10, 11], which can be useful in assessing genetic variability and for establishing genetic distances and phylogenetic relationships between organism lines, species and genus [12]. In recent years, such approaches have established the distance between different plant species and ecotypes, as achieved for Brassicaceae [13], wheat [14], *Arabidopsis* [8, 15], coffee [16] and potato [17].

The present work was undertaken to decipher the lentil seed protein composition, to characterize local lentil genetic resources and to confirm the potential of proteomics to investigate the relationships among and within different plant landraces. To accomplish this aim, first a lentil seed proteome reference map was produced identifying the majority of the most abundant proteins. Then, a multivariate statistical analysis was accomplished to compare the seed proteome of different populations, identifying protein markers that discriminate the different landraces.

2 Materials and methods

2.1 Plant material

Lentil mature seeds of 12 lentil populations were provided by the Molise Germoplasm Bank at the University of Molise (Pesche), Italy; 7 of them (6 microsperma and 1 macrosperm) were from Capracotta, a small village in the Molise region (Central Italy) and 5 were from commonly marketed (Turca Rossa, Canadese and Castelluccio di Norcia) or autochthon landraces (Rascino and Colfiorito). Castelluccio di Norcia is the unique Italian variety to be awarded with the Geographically Protected Brand (IGP). All the *L. culinaris* landraces used in this work have been previously characterized at genetic and morphological levels [4]. Two biological replicates consisting of almost 90 seeds (2.0 g) were analyzed for each lentil population.

2.2 Protein extraction

Total proteins were extracted according to the method of Rabilloud [18] with minor modifications. Independent samples (2.0 g of dry seeds) were finely powdered in liquid N_2, and the resulting material was suspended in 10 mL of 10% TCA, 0.07% β-mercaptoethanol, 1 mM PMSF in cold acetone at −20°C and then filtered through Miracloth. Proteins were precipitated at −20°C for 4 h, centrifuged at 35 000 × *g* for 15 min and rinsed twice with cold acetone, 0.07% β-mercaptoethanol, 1 mM PMSF for 4 h at −20°C. The pellet was recovered by centrifuging at 35 000 × *g* for 15 min, dried under vacuum and solubilized in 300 µL of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% v/v Triton X-100, 20 mM Tris-HCl, 1% w/v DTT, containing 0.2% w/v ampholine 3–10 and 0.15% w/v ampholine 5–7. Protein concentration was estimated according to a modified Lowry's method using BSA as standard [19].

2.3 2-DE

Protein pellets were washed with ice-cold methanol (once) and ice-cold acetone (three times), dried under reduced pressure and dissolved in IEF buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT, 1% w/v carrier ampholytes pH 5–8; Bio-Rad, Hercules, CA, USA). IPG strips (18 cm pH 5–8, ReadyStrip, Bio-Rad) were rehydrated overnight with 460 µL of IEF buffer containing 400 µg of total proteins. Proteins were focused using a Protean IEF Cell (Bio-Rad) at 12°C, applying the following voltages: 250 V (90 min), 500 V (90 min), 1000 V (180 min) and 8000 V for a total of 55 kV h [20]. After focusing, the proteins were reduced by incubating the IPG strips with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of 50 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of bromophenol blue for 15 min. Electrophoresis in the second dimension was carried out on 12% polyacrylamide gels (18 × 24 cm × 1 mm) with a Protean apparatus (Bio-Rad) in 25 mM Tris-HCl, pH 8.3, 1.92 M glycine and 1% w/v SDS, with 120 V applied for 12 h, until the dye front reached the bottom of the gel. 2-DE gels were stained with colloidal Coomassie G250. Samples were run in triplicate.

2.4 Image acquisition and analysis

2-DE gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Image analysis was performed using the PDQuest software (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for three different replicates of each biological sample, and statistical Student's *t*-test analysis ($P<0.01$) was performed to find out significant changes in protein folding between samples. A two-fold change in normalized spot densities was considered indicative of a differential expression.

2.5 In gel digestion and mass spectrometry

Spots were manually excised from gels and digested with trypsin, as previously reported [21]. Digest aliquots were

removed and subjected to a desalting/concentration step on μZipTipC18 (Millipore, Bedford, MA, USA) using ACN as eluent before MALDI-TOF-MS or nanoLC-ESI-LIT-MS/MS analysis.

In the first case, peptide mixtures were loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic acid as matrix, and analyzed by using Voyager DE PRO and STR mass spectrometers (Applied Biosystems, Framingham, MA, USA), operating in positive ion reflectron mode, with an acceleration voltage of 20 kV, a nitrogen laser of 337 nm and a laser repetition rate of 4 Hz. The final mass spectra, measured over a mass range of 800–4000 Da and by averaging 50–300 laser shots, were elaborated using the DataExplorer 5.1 software (Applied Biosystems) and manually inspected to get the corresponding peak lists. Internal mass calibration was performed with peptides deriving from trypsin autoproteolysis.

In the second case, the digests were analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (Thermo) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon). Peptide mixtures were separated on an Easy C_{18} column (10×0.075 mm, 3 μm; Proxeon) using a linear gradient from 5 to 50% of ACN in 0.1% formic acid, over 60 min, at a flow rate of 300 nL/min. Spectra were acquired in the range of m/z 300–1800. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 3 min). The mass isolation window and collision energy were set to m/z 3 and 35%, respectively.

2.6 Protein identification

MASCOT software package was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2008/05/03) in MALDI-TOF peptide mass fingerprinting experiments by using a mass tolerance value of 40–80 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. Candidates with a MASCOT score >64, corresponding to $p<0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

Sequest software package was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2008/05/03) in ESI-LIT-MS/MS experiments by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. Candidates with more than two assigned peptides were ranked in ascending order according to consensus scores and false positive identifications minimized by filtration against four of the following criteria: Xcorr >2, ΔCn >0.2, Sp >400, rsp <5, ions $>30\%$ [22]. Where appropriate, protein identification was checked manually to provide for a false positive rate of $<1\%$ using Xcorr and ΔCn values described and validated elsewhere [23], and further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

2.7 Statistical analysis

Differentially expressed proteins among Capracotta 1 and all the other lentil populations were subjected to multivariate statistical analyses by using the software PAST [24]. Principal component analysis (PCA) was carried out on correlation distance matrix; confidential ellipse ($P = 95\%$) was computed for lentils from Capracotta, and protein coefficients of Principal Component 1 (PC1) and Principal Component 2 (PC2) were summarized in a structure matrix to individuate more significant proteins. Classification analysis was computed by means of neighbor-joining method [25], on the Euclidean distance matrix computed on the 103 protein spots; a phenogram was built-up, and bootstrap values were computed on 999 random permutations.

3 Results

The availability of a 2-DE reference map of lentil seeds is a primary requisite to study the natural diversity in various *L. culinaris* ecotypes. Thus, we generated proteome reference maps of mature lentil seeds by analysing with 2-DE whole-protein extracts from 12 plant populations (representative gels are shown in Fig. S1 in Supporting Information). These 2-DE maps (six in number), produced in triplicate for each of the two independent protein extraction experiments, showed a high level of reproducibility. A master gel containing spots observed in all run replicates was firstly created for each landrace; thereafter, average gels were matched by using the Capracotta 1 landrace as reference (Fig. 1). Average proteomic maps showed 300–350 well-resolved spots for Capracotta populations and 230–270 in the case of commercial varieties. The majority of spots detected in these maps showed analogous positions and intensities, as indicated by the degree of gel similarity between the various ecotypes and the reference landrace (within a 75–55% range; Fig. S1 in Supporting Information). A clickable Capracotta 1 landrace map website was also generated, which is now available through the World Wide Web network at the URL address (http://www.bancagermoplasmamolise.unimol.it). This map presents almost 350 spots, ranging in a pI value 5–8 and M_r value 9–120 kDa.

A software-assisted comparison of relative spot densities between the Capracotta 1 and the other lentil populations revealed 103 protein spots as differentially expressed

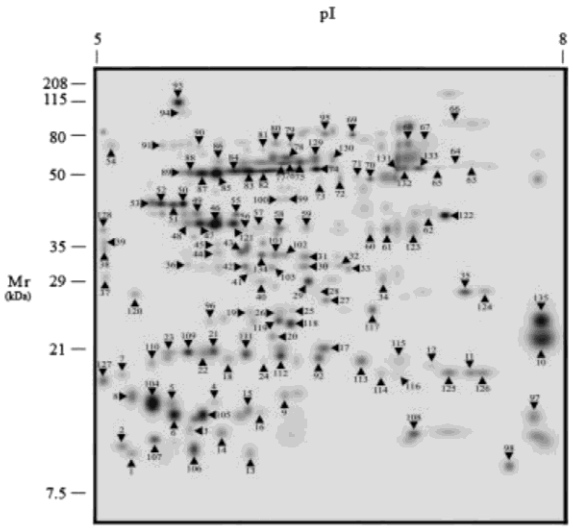

Figure 1. 2-DE reference map of *L. culinaris* mature seeds. Proteins were resolved on pH 5–8 IPGs (first dimension) and 12% SDS-PAGE (second dimension) and were visualized by colloidal Coomassie blue staining. Spot numbering refers to Table S1 in the Supporting Information reporting protein identification as ascertained by MS analysis.

($p < 0.05$) among and within landraces, whereas the remaining ones showed a constant expression (Supporting Information Table S1). Variable and most abundant constant protein spots (135 in number) were excised from the gel and digested for further analysis by MALDI-TOF PMF and/or nanoLC-ESI-LIT-MS/MS; 215 proteins were identified, which corresponded to 58 unique sequence accessions. As reported in Supporting Information Table S1, 82 spots showed unequivocal identification defined by 39 different accessions, 41 spots revealed multiple identification and 12 spots remained unidentified, probably as a result of the poor information available on *L. culinaris* genome. We believe that the absence of a specific sequence counterparts within the available lentil genome was again the reason for the multiple identification of homologous proteins from different plants for a number of analyzed spots.

Based on Bevan's classification [26], the identified polypeptides were grouped into eight different functional classes (Fig. 2A); storage proteins were the most represented group (79%), followed by proteins involved in disease/defense (13.5%), transcription (1.8%), cell growth/division (1.4%), energy (1.4%), metabolism (1%), protein synthesis (0.5%) and others (1.4%). The majority of proteins present in the lentil map corresponded to the storage 7S (72%) and 11S (21%) globulins (Fig. 2B, Supporting Information Table S1). Together with the other storage proteins, they accounted for 0.82 and 0.64 of the mean normalized spot volume with respect to all identified spots and all spots present in the map, respectively.

The 7S storage proteins identified on the map were mainly represented by different vicilin (34 spots), provicilin (12 spots) and vicilin 47k (6 spots) isoforms, with M_r 15–59 kDa and p*I* 5.8–6.6, identified with sequences from other plants (Fig. 1, Supporting Information Table S1); 29 and 19 spots were also associated to lentil allergen Len c1.0101 and Len c1.0102, respectively, with M_r values ranging from 14 to 39 kDa and p*I* values from 5.3 to 7.16. Belonging to this group were also convicilin isoforms (13 spots) with M_r 10–69.5 kDa and p*I* 5–7.2, which were identified with two accessions from lentil and one from *Vicia narbonensis*. The 11S storage proteins were legumins (16 spots) with M_r values ranging from 38 to 43 kDa and p*I* values from 5.3 to 5.9, always identified with sequences from other legumes (Fig. 1, Supporting Information Table S1). Two spots were corresponded to a single sequence entry, whereas the remaining were associated to multiple accessions. A single spot was identified as soybean β-conglycinin, another 11S globulin related in sequence to legumins. The remaining storage proteins were other cupin-like proteins (four spots) with M_r 18.2–40.6 kDa and p*I* 5.1–6, associated with two accessions, and 2S albumins (four spots) with M_r 29.5–31.56 kDa and p*I* 5.9–6.8, identified as two accessions; in these cases, identified sequences were always from other plant species.

The 103 differentially expressed proteins among the Capracotta 1 and all the other populations were subjected to multivariate statistical analysis for ordination and classification. PCA results showed that PC1 and PC2 explained 36.99 and 16.32% of total variance, respectively. Along PC1, the populations of Capracotta, with exception of Capracotta 7, were distinguished from all the five commercial landraces (Fig. 3). Confidential ellipse computed from lentil populations from Capracotta explained the probability (95%) of each specimen to belong to Capracotta group. Furthermore, Fig. 3 shows that all Capracotta populations were distinguishable from the commercial lentils, with the exception of Castelluccio di Norcia, which grouped within the six populations from Capracotta.

The analysis of PC1 coefficients provided information on the weight of the each protein on the discrimination of different landraces. Among the 103 proteins differentially expressed, 24 resulted more indicative in the PCA (Supporting Information Table S2). The remaining 79 showed coefficient values between −0.1 and 0.1, and their weight on the PC1 and PC2 was not discriminative among landraces. In detail, nine proteins spots (spots 5, 6, 10, 15, 20, 35, 92, 97 and 98), showing a negative coefficient on PC1, were highly correlated in separating Capracotta lentils from the commercial varieties on the PC1 (Supporting Information Table S2). As shown in Fig. 4, these proteins (in particular, spots 10, 97 and 35) were more abundant in Capracotta populations. On the contrary, six protein spots (49, 51, 52, 53, 56 and 77), with positive coefficients on PC1, characterized Capracotta for their lower expression (Fig. 4). On the PC2, protein spots 46, 50, 51, 52, 53, 56, 57, 63, 65, 68, 83, 85 and 87 were characterizing for the commercial lentils (Supporting Information Table S2). In particular, protein spots with the highest negative coefficients of PC2 were significant in the separation of Turca Rossa from the

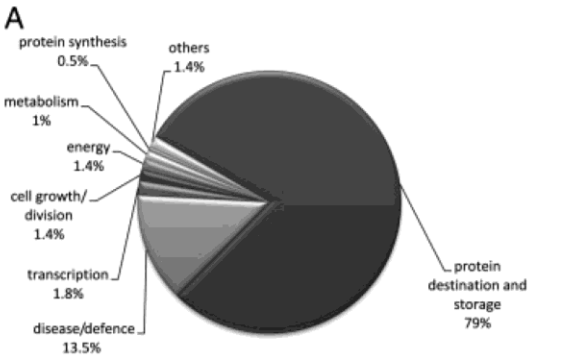

Figure 2. (A) Functional classification of the proteins identified in mature lentil seeds (number of spots). (B) Relative percentage of 7S and 11S globulins and other proteins within the storage protein group (number of spots).

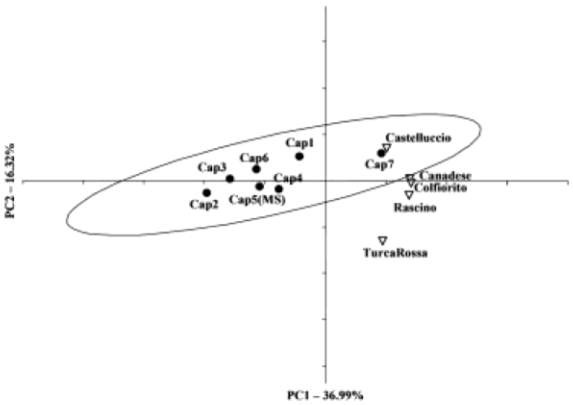

Figure 3. Scatter plot of the first two principal components from the PCA performed on a correlation matrix computed on the 103 variable protein spots (quantitative data).

other populations. However, protein spots 56 and 63 were abundant also in the Canadese landrace, whereas protein 51 characterized Turca Rossa and Rascino; the latter was also distinguished by spot 46 (Fig. 4, Supporting Information Table S2). Protein spot 5, with positive value on PC2 (Supporting Information Table S2), distinguished Capracotta, as it was absent in all the commercial varieties except Castelluccio di Norcia (Fig. 4).

PCA was also carried out to analyse the seed proteome variability within the seven Capracotta populations. The results show that altogether PC1, PC2 and PC3 explained 70.48% of total variance. By comparing scatter plots (Fig. 5) and table of coefficients extracted from PCA, along PC1, it resulted that population Capracotta 7 was characterized by the lower quantity of protein 10 with respect to other Capracotta populations (Supporting Information Table S3). Along PC2, protein spots 46, 78, 59, 80 and 50 characterized population Capracotta 4 from all the other ones; along PC3, protein spots 17, 20, 57, and 92 were typical for population Capracotta 3, whereas protein spot 67 resulted indicative for Capracotta 6.

Euclidean distance matrix computed for the 103 variable proteins was subjected to neighbor-joining analysis (classification). A significant dendogram was obtained, showing the seven populations from Capracotta grouped and distinguished from all the other commercial lentils at significant level (100%) of bootstrap (Fig. 6). The exception is represented by Castelluccio di Norcia landrace, which grouped with the populations from Capracotta and, in particular, with population Capracotta 7 (with a bootstrap level of 71%).

4 Discussion

This study originally describes a detailed 2-DE-based proteomic analysis of mature lentil seeds, where a total of 122 protein spots were identified as matching to 215 protein entries, which corresponded to 58 unique accessions (Supporting Information Table S1). The lack of recognition of several spots (12 in number) and the multiple identification of similar proteins from different plant species were both ascribable to the poor information present on *L. culinaris* genome. This contrasts with the other proteomic studies on other plant seeds, *i.e. Arabidopsis thaliana* [27–29], *Medicago truncatula* [30], *Triticum aestivum* [31–33], *Hordeum vulgare* [34, 35], *Glycine max* [36] and *Pisum sativum* [37], for which a broad covering of the whole genome was present. Nevertheless, MS/MS experiments ensured a positive identification of most (56%) of the analyzed lentil spots, mainly associating it to sequence entries from other legume species.

Proteins identified in this work were classified into different functional categories (Fig. 2A), which will be discussed in the following sections; storage proteins were the most represented group, followed in order by proteins involved in disease/defense, transcription, cell growth/division, energy, metabolism, protein synthesis and others.

4.1 Storage proteins

Seed storage proteins have been functionally associated in providing the seedling with nitrogen, carbon and sulfur during plant germination [38]. They have been reported also having a role in defending seeds against bruchids and pathogens [39, 40]. As originally reported in preliminary studies, 7S (vicilins and convicilins) and 11S (legumins) globulins represent the major storage proteins in legume

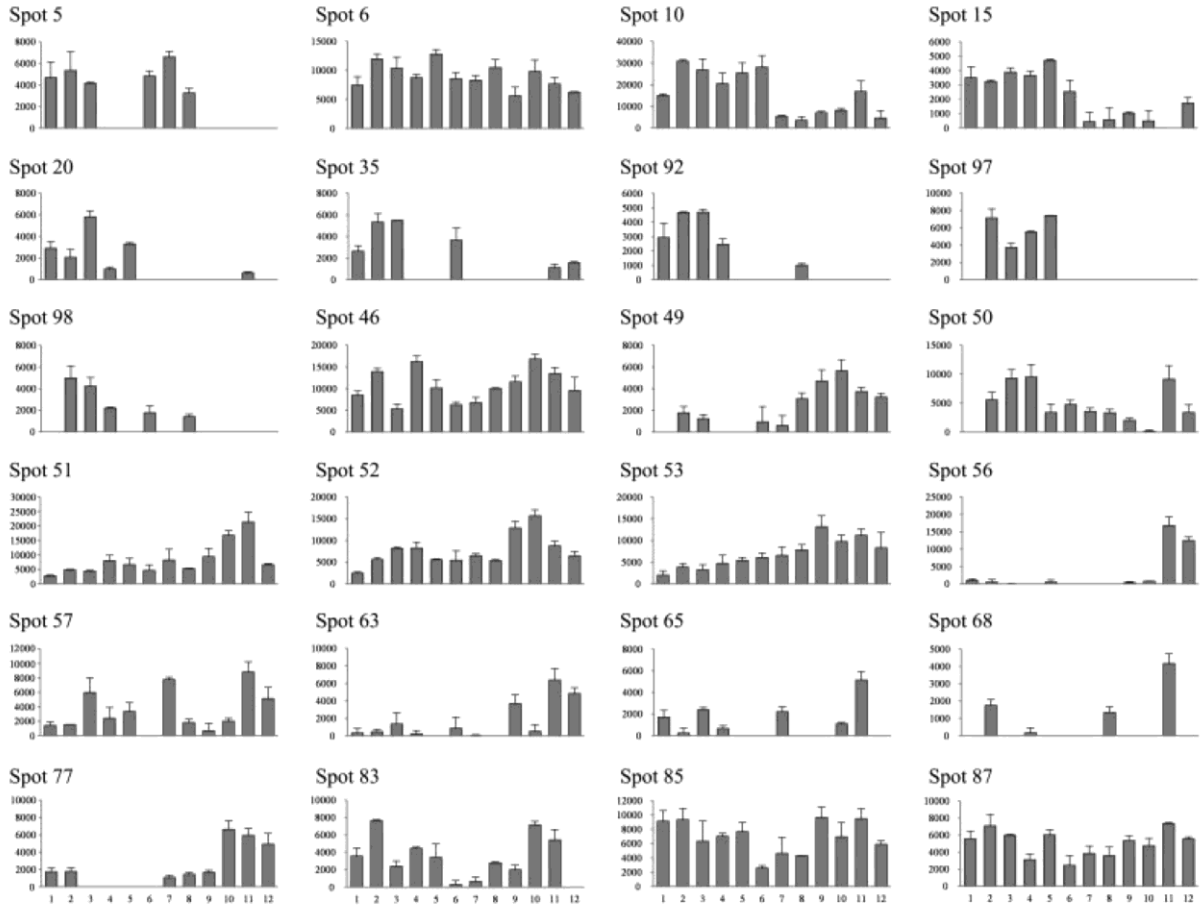

Figure 4. Differential expression of the 24 protein spots that resulted more indicative in discriminating the different landraces, according to the PCA. Proteins were considered as differentially expressed when a relative fold change >2.0 or <0.5 was measured. 1–7 Capracotta; 8 Castelluccio di Norcia; 9 Colfiorito; 10 Rascino; 11 Turca Rossa; 12 Canadese.

seeds [41];] these findings were confirmed later on by proteomic analysis on *M. truncatula* [30], *G. max* [36] and *P. sativum* [37]. Storage proteins identified in lentil seeds were similarly well represented by abundant 7S and 11S globulins; our analysis revealed for these proteins at a high level of complexity in terms of spot number and distribution of their pI/M_r values (Fig. 1, Supporting Information Table S1). Similarly to pea and contrarily to other legumes [37], the number of spots identified as 7S proteins was markedly higher compared with the number of the 11S globulins (Supporting Information Table S1). The 7S/11S quantitative ratio in lentil seeds was determined as close to 2.78, whereas it is almost 1 and 0.25 in *P. sativum*and*M. truncatula*, respectively. Because the 7S/11S ratio is an important determinant of the seed nutritional quality [37], the particular nature of lentil seed protein composition had to be precisely analyzed and may provide specific end-uses for its proteins.

In particular, lentil 7S globulins were characterized by the highest degree of heterogeneity. Capracotta 1 2-DE map showed the occurrence of 34 vicilins, 12 provicilins, six vicilins 47k, 13 convicilins, and 29 and 19 isoforms of allergen Len c1.0101 and Len c1.0102, respectively (Fig. 1, Supporting Information Table S1). 7S globulins have been poorly investigated in *L. culinaris* and scant are the information available in the literature. A genomic study proposed that vicilins are encoded at least by four types of gene sequences, forming two small multigene subfamilies having a M_r value of 45 and 50 kDa [42]. Moreover, several lentil seed allergens (Len c1.0101 and c1.0102) have been cloned and classified as vicilins/provicilins based on their high sequence homology with other legume species [43, 44]. These 7S globulins, together with other proteins with a higher M_r value, have been identified as major allergens in lentil, a plant food causing widespread allergic reactions in pediatric patients from Mediterranean and Asian areas [43].

Vicilins have been widely studied in *P. sativum*, where subunits with 50, 34–30, 25–18, 16, 13 and 12 kDa resulted prominent components in SDS-PAGE and 2-DE analysis of seed extracts [37]. Vicilin heterogeneity has been also reported in other plant species [27–36] and has been attrib-

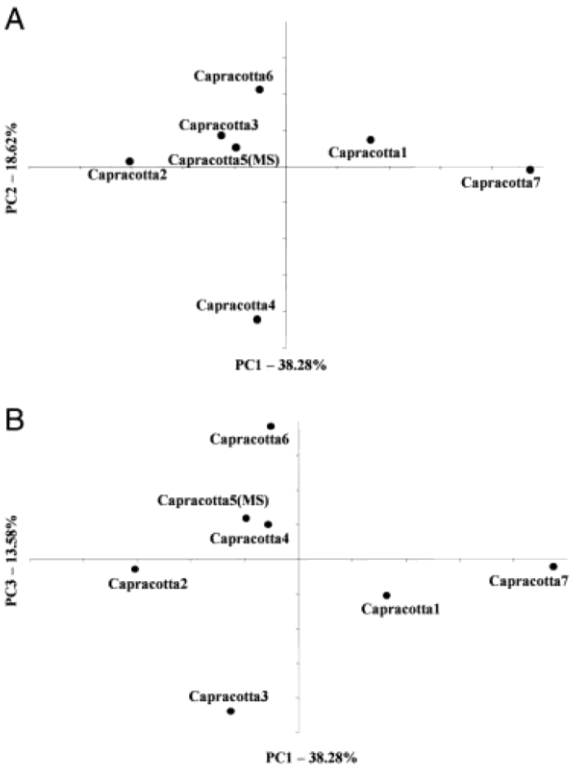

Figure 5. Scatter plots of the first three principal components from the PCA performed on a correlation matrix computed on the 103 variable protein spots (quantitative data). Capracotta 5 (MS) corresponds to the macrosperm. Panels A and B show the location of Capracotta populations in the PC1-PC2 and PC1-PC3 scatter plots, respectively.

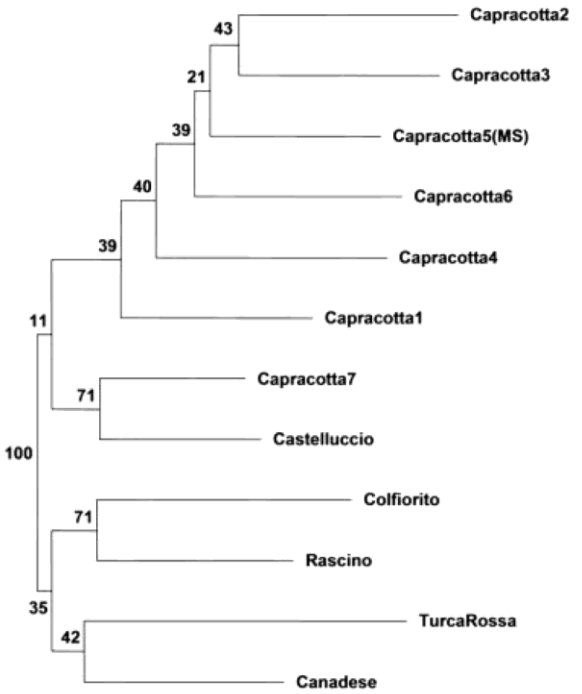

Figure 6. Dendogram computed from the Euclidean distance matrix calculated on the 103 variable protein spots. Bootstrap values were computed on 999 random permutations.

uted to: (i) expression of multigene families encoding for different primary sequences; (ii) differential proteolytic processing of expressed genes; and (iii) differential protein glycosylation [45–48]. These events likely account for the heterogeneity also observed for lentil 7S globulins. Obviously, the lack of information on *L. culinaris* genome represented a strong limitation for an exhaustive elucidation of the complexity of this globulin fraction.

On the contrary, 16 spots were identified as 11S legumins (Supporting Information Table S1). As no sequence data are available for lentil legumins, identification was obtained on the basis of protein counterparts from other plant species. Legumins are storage polypeptides of 60 kDa, showing sequence and structural analogies with vicilins [38]. These proteins have also been reported to act as allergens in different foods [49–51]. They occur as acidic (40 kDa) and basic (20 kDa) polypeptides following disulfide bond reduction. Based on measured M_r and p*I* values, we verified the occurrence of both polypeptides in the 2-DE maps of lentil seeds, similarly to what already reported for *G. max* [52], *V. faba* [53] and *P. sativum* [37].

Other spots in the lentil 2-DE map were associated with storage proteins. Four spots were identified as two RmlC-type cupin accessions, and four spots as two 2S albumin accessions. Although these proteins are generally classified as storage proteins, their function has also been associated to germination and stress defense functions [54]. Some of these proteins have also been reported to determine allergenicity in legume-based foods [49].

4.2 Proteins potentially involved in seed conservation and germination

In dry mature seeds, which are storage and metabolic dormant tissues, the overwhelming presence of storage proteins frequently obscures the occurrence of other enzymes involved in different metabolic processes. In this work, proteins involved in stress defense, metabolic and regulatory functions have also been identified. A number of lentil seed spots corresponded to proteins involved in abiotic and biotic stress responses (Fig. 2, Supporting Information Table S1). In particular, we revealed that the occurrence of catalase (spot 62), which reduces H_2O_2 concentration, has a seed glyoxysome localization [55, 56] and is essential for acquisition of embryos desiccation tolerance [57, 58]. Other reactive oxygen species scavenging enzymes involved in the dehydration tolerance were also identified as dehydroascorbate reductase (spot 28), aldo/keto reductase (spot 100) and Mn-SOD (spot 117) [59, 60]. These proteins also exert their protective role in metabolic/developmental processes associated with the preparation for germination [61]. In this

context, ferritin (spot 40) was recently demonstrated to protect *Arabidopsis* against oxidative damage by limiting reactive oxygen species levels and facilitating seed germination [62]. Acquisition of seed desiccation tolerance seems also ensured in lentil seeds by two heat shock proteins (spots 94 and 126), TAG-associated factor/alcohol dehydrogenase (spot 123) and P54 protein (spots 26, 70, 85, 87–89 and 99) [63]. The latter protein seems to exert a protective function of the chromatin structure against desiccation [64, 65]. Proteins that became abundant in seed embryos and may control embryogenesis, such as Em protein (spot 16), seed maturation protein (spot 39) and cyclin-like F-box (spot 92), were also identified [66, 67]. Other proteins seem to ensure lentil seed response to biotic stresses. Six spots were identified as four lectin accessions (three from *L. culinaris*). Because of their binding to bacterial polysaccharides, these proteins have been associated with plant response against pathogens, being also involved in seed germination/conservation [68, 69]. Similarly, the Bowman–Birk protease inhibitor observed in the lentil map (spot 98) may ensure a defense mechanism of the seed against insect midgut proteases [70]. Findings on the involvement of this molecule in the prevention of tumorigenesis suggest a possible positive contribution of protease inhibitors to the nutritional value of legume seeds [71, 72].

A second family included proteins involved in energy metabolism and nutrients production. Spot 81 (enolase), 120 (β-galactosidase), 35 (probable proteinase) and 90 (F1 ATPase) corresponded to enzymes essential for efficient (poly)saccharide or protein degradation and ATP production. This demonstrates that the seeds are well-equipped in quickly releasing stored energy and nutrients (storage proteins) for rapid growth of plant tissues, as during the early germination steps [73, 74], and contain stored enzymes to allow a rapid resumption of essential metabolic activities.

Other non-storage proteins identified were enzymes involved in DNA replication, such as ATP-binding DNA-directed polymerase (spot 2) and predicted protein DNA ligase IV (spot 9). The occurrence of these proteins in seeds is not surprising, as the re-activation of a reduced number of core cell cycle proteins, which trigger DNA replication, but are not sufficient to drive cells into mitosis, provides minimal cell cycle machineries for seed germination [75].

The last class of non-storage proteins included proteins involved in various physiological processes and housekeeping proteins. Spots 131–133 were identified as IAP1, the most abundant protein covalently modified by the phytohormone indoleacetic acid [76]. Expression and modification of IAP1 is correlated to the developmental period of rapid growth during seed development, whereas its degradation occurs during germination. Indoleacetic acid -modified proteins represent a distinct class of conjugated phytohormones and appear to be the major form of auxin in seeds [77]. Other identified spots were proteins involved in protein (spots 37, 39, 63, 64, 65 and 129) and polysaccharide (spot 86) biosyntheses.

4.3 Seed proteome variations within and between ecotypes

Local genetic plant resources, characterized by a wide genetic variability and high adaptation to different environmental conditions, are more subjected to genetic erosion risks, especially when its conservation is almost entirely deputed to elderly people. In Italy, a combination of different geographical characteristics allowed the evolution of several lentil landraces, which were investigated in a previous study based on genetic and morphological observations [4]. The correlation found between the genetic and morphological data allowed us to distinguish specific landraces. Previous studies have also used SDS-PAGE-based protein analysis to evaluate the genetic diversity between various lentil populations [78–80].

In this work, we used a 2-DE-based proteomic analysis of mature lentil seeds to identify specific protein markers of different ecotypes. Reference maps were obtained for 12 lentil populations belonging to local landraces (Capracotta) and five commercial varieties (Turca Rossa, Canadese, Castelluccio di Norcia, Rascino and Colfiorito). A comparison of these maps revealed 103 protein spots as differentially expressed within and between populations. Multivariate statistical analysis carried out on variably expressed spots showed that 24 protein components, namely 7S and 11S globulins, maturase K, P54 protein, probable proteinase, cyclin-like F-box and Bowman–Birk protease inhibitor, were essential for population discrimination (Supporting Information Table S2), thus proposing it as landrace markers. PCA analysis revealed that Capracotta lentil seeds were well distinguished (95%) from all the other commercial varieties, with the exception of Castelluccio di Norcia, which has also been reported to share genetic/morphological features with Capracotta populations [4] (Fig. 4, Supporting Information Table S2). Confidential ellipse and neighbor-joining representations clearly showed that the Capracotta populations grouped together and distinguished from the other commercial varieties (with a bootstrap value of 100%; Figs. 3 and 6). The exception again was represented by Castelluccio di Norcia landrace, which grouped with the Capracotta 7 population. The similarity between Capracotta and Castelluccio di Norcia landraces may be attributed to a common geographical origin, as these landraces are cultivated in close central Italian areas.

In particular, nine spots associated with 7S globulins (5 in number), legumin (1), cyclin-like F-box (1), probable proteinase (1) and Bowman–Birk protease inhibitor (1) were particularly abundant and highly correlated in characterizing Capracotta populations, which also showed poor legumin levels (Fig. 4, Supporting Information Table S2). In contrast, 13 spots characterized Turca Rossa, Canadese and Rascino landraces, which showed high concentration of 11S globulins (five spots), 7S globulins (four spots), maturase K (two spots) and P54 protein (two spots) (Fig. 4, Supporting Information Table S2). All together, these data suggest that the lentil ecotypes here investigated are characterized by

different levels of proteins involved in abiotic and biotic stress responses, which are probably the result of natural or human selection toward specific adaptive genetic traits, *i.e.* ability to resist to the challenging environmental conditions (Capracotta landraces) or a reduced allergens content (commercial varieties).

5 Concluding remarks

In this work, the lentil seed protein composition has been deciphered, with the identification of most abundant protein components. Similarly to other legumes, the lentil seed proteome is mainly represented by a high heterogeneity of storage proteins; a number of other proteins potentially involved in seed conservation and germination was also observed. Our results suggest that lentil seeds have developed a ''survival kit'' allowing it (i) to face various abiotic/biotic stresses, (ii) to provide various nutritional protein and non-protein compounds for the growing plantlet, and (iii) to enhance germination by ready-protein machineries present in the seed. Most of these proteins were also found in seeds from other species [27–37]. This study further confirms that proteomic approaches coupled with multivariate statistical analysis can be used to asses genetic distances and phylogenetic relationships between various plant species and ecotypes [8, 12–17].

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6 References

[1] Zohary, D., *Econ. Bot.* 1972, *26*, 326–332.

[2] Ford, R. R., Taylor, P. W. J., *Theor. Appl. Genet.* 2003, *107*, 910–916.

[3] Erskine, W., *Euphytica* 1997, *93*, 107–112.

[4] Scippa, G. S., Trupiano, D., Rocco, M., Viscosi, V., Di Michele, M., D'Andrea, A., Chiatante, D., *Heredity* 2008, *101*, 136–144.

[5] Ladizinsky, G., Abbo, S., *Genet. Resour. Crop Evol.* 1993, *39*, 51–54.

[6] Piergiovanni, A. R., *Genet. Resour. Crop Evol.* 2000, *47*, 305–314.

[7] Park, O. K., *J. Biochem. Mol. Biol.* 2004, *37*, 133–138.

[8] Chevalier, F., Martin, O., Rofidal, V., Devauchelle, A. D., Barteau, S., Sommerer, N., Rossignol, M., *Proteomics* 2004, *4*, 1372–1381.

[9] David, J. L., Zivy, M., Cardin, M. L., Brabant, P., *Theor. Appl. Genet.* 1997, *95*, 932–941.

[10] Damerval, C., Vartanian, N., de Vienne, D., *Plant Physiol.* 1988, *86*, 1304–1309.

[11] de Vienne, D., Burstin, J., Gerber, S., Leonardi, A., Le Guilloux, M., Murigneux, A., Beckert, M. *et al.*,*Heredity* 1996, *76*, 166–177.

[12] Thiellement, H., Bahrman, N., Damerval, C., Plomion, C., Rossignol, M., Santoni, V., de Vienne, D. *et al.*, *Electrophoresis* 1999, *20*, 2013–2026.

[13] Marqués, K., Sarzin, B., Chané-Favre, L., Zivy, M., Thiellement, H., *Proteomics* 2001, *1*, 1457–1462.

[14] Jacobsen, S., Nesic, L., Petersen, M., Sondergaard, I., *Electrophoresis* 2001, *22*, 1242–1245.

[15] Ruebelt, M. C., Leimgruber, N. K., Lipp, M., Reynolds, T. L., Nemeth, M. A., Astwood, J. D., Engel, K.-H. *et al.*, *J. Agric. Food Chem.* 2006, *54*, 2162–2168.

[16] Gil-Agusti, M. T., Campostrini, N., Zolla, L., Ciambella, C., Invernizzi, C., Righetti, P. G., *Proteomics* 2005, *5*, 710–718.

[17] Lehesranta, S. J., Davies, H. V., Shepherd, L. V. T., Nunan, N., McNicol, J. W., Auriola, S., Koistinen, K. M. *et al.*, *Plant Physiol.* 2005, *138*, 1690–1699.

[18] Rabilloud, T., *Proteome Research: Two-dimensional Gel Electrophoresis and Identification Methods, Principles and Practice*, Springer, Berlin Heidelberg, Germany 2000.

[19] Peterson, G. L., *Anal. Biochem.* 1977, *83*, 346–356.

[20] Rocco, M., D'Ambrosio, C., Arena, S., Faurobert, M., Scaloni, A., Marra, M., *Proteomics* 2006, *6*, 3781–3791.

[21] Talamo, F., D'Ambrosio, C., Arena, S., Del Vecchio, P., Ledda, L., Zehender, G., Ferrara, L. *et al.*, *Proteomics* 2003, *3*, 440–460.

[22] Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M., Yates, J. R., *Nat. Biotechnol.* 1999, *17*, 676–682.

[23] Peng, J., Elias, J. E., Thoreen, C. C., Licklider, L. J., Gygi, S. P., *J. Proteome Res.* 2003, *2*, 43–50.

[24] Hammer, M. F., Karafet, M. T., Redd, A. J., Jarjanazi, H., Santachiara-Benerecetti, A. S., Soodyall, H., Zegura, S. L., *Mol. Biol. Evol.* 2001, *18*, 1189–1203.

[25] Saitou, N., Nei, M., *Mol. Biol. Evol.* 1987, *4*, 406–425.

[26] Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R. *et al.*, *Nature* 1998, *391*, 485–488.

[27] Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., Job, D., *Plant Physiol.* 2001, *126*, 835–848.

[28] Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., Job, D., *Plant Physiol.* 2002, *129*, 823–837.

[29] Gallardo, K., Job, C., Groot, S. P. C., Puype, M., Demol, H., Vandekerckhove, J., Job, D., *Plant Physiol.* 2002, *116*, 238–247.

[30] Gallardo, K., Le Signor, C., Vandekerckhove, J., Thompson, R. D., Burstin, J., *Plant Physiol.* 2003, *133*, 664–682.

[31] Skylas, D. J., Copeland, L., Rathmell, W. G., *Proteomics* 2001, *1*, 1542–1546.

[32] Islam, N., Woo, S. H., Tsujimotp, H., Kawasaki, H., Hirono, H., *Proteomics* 2002, *2*, 1146–1155.

[33] Majoul, T., Bancel, E., Tribo, E., Hamida, J. B., Branlard, G., *Proteomics* 2004, *4*, 505–513.

[34] Finnie, C., Melchior, S., Roepstorff, P., Svensson, B., *Plant Physiol.* 2002, *129*, 1308–1319.

[35] Østergaard, O., Finnie, C., Laugesen, S., Roepstorff, P., Svensson, B., *Proteomics* 2004, *4*, 2437–2447.

[36] Hajduch, M., Ganapathy, A., Stein, J. W., Thelen, J. J., *Plant Physiol.* 2005, *137*, 1397–1419.

[37] Bourgeois, M., Jacquin, F., Savois, V., Sommerer, N., Labas, V., Henry, C., Burstin, J., *Proteomics* 2009, *9*, 254–271.

[38] Shewry, P. R., Napier, J. N., Tatham, A. S., *Plant Cell* 1995, *7*, 945–956.

[39] Sales, M. P., Gerhardt, I. R., Grossi de Sa, F., XavierFilho, J., *Plant Physiol.* 2000, *124*, 515–522.

[40] Yamada, K., Shimada, T., Kondo, M., Nishimura, M., Hara-Nishimura, I., *J. Biol. Chem.* 1999, *274*, 2563–2570.

[41] Croy, R. R. D., Gatehouse, J. A., Evans, I. M., Boulter, D., *Planta* 1980, *148*, 49–56.

[42] Saenz de Miera, L. E., Perez de la Vega, M., *Mol. Biol. Evol.* 1998, *15*, 303–311.

[43] Sanchez-Mouge, R., Blanco, C., Diaz-Perales, A. D., *J. Allergy Clin. Immunol.* 2000, *103*, 514–519.

[44] Lopez-Torrejon, G., Ibanez, M. D., Ahrazem, O., *Clin. Exp. Allergy* 2003, *33*, 7–13.

[45] Casey, R., Downey, C., Ellis, N., *Oxford Surv. Plant Mol. Cell Biol.* 1986, *3*, 1–95.

[46] Derebyshire, E., Wright, D. J., Bowlter, D., *Phytochemistry* 1976, *15*, 3–24.

[47] Nielsen, N. C., Dickinson, C. D., Cho, T., Thanh, V. H., Scallon, B. J., Fischer, R. L., Sims, T. L. *et al.*, *Plant Cell* 1989, *1*, 313–328.

[48] Wright, R., Basson, M., D'Ari, L., Rine, J., *J. Cell Biol.* 1988, *107*, 101–114.

[49] Breiteneder, H., Ebner, C., *J. Allergy Clin. Immunol.* 2004, *113*, 821–830.

[50] Bässler, O. Y., Weiss, J., Wienkoop, S., Lehmann, K., Scheler, C., Dölle, S., Schwarz, D. *et al.*, *J. Proteome Res.* 2009, *8*, 1111–1122.

[51] Comstock, S. S., Robotham, J. M., Tawde, P., Kshirsagar, H., Sathe, S. K., Roux, K. H., Teuber, S. S., *J. Agric. Food Chem.* 2008, *56*, 5977–5982.

[52] Nielsen, N. C., in: Altschul, A. M., Wilcke, H. L. (Eds.), *Structure of Soy Proteins. In New Protein Foods*, Academic Press, New York 1985, pp. 27–64.

[53] Wright, D. J., Boulter, D., *Biochem. J.* 1974, *141*, 413–418.

[54] Agizzio, A. P., Carvalho, A. O. R., Suzanna de Fátima, F., Machado, O. L. T., Alves, E. W., Okorokov, L. A., Samarão, S. S. *et al.*, *Arch. Biochem. Biophys.* 2003, *416*, 188–195.

[55] Willekens, H., Inzé, D., Van Montagu, M., Van Camp, W., *Mol. Breed.* 1995, *1*, 207–228.

[56] Scandalios, J. G., Guan, L., Polidoros, A. N., *Cold Spring Harbor Laboratory*, Cold Spring Harbor, New York 1997, pp. 343–406.

[57] Bailly, C., Audigier, C., Ladonne, F., Wagner, M. H., Coste, F., Coubineau, F., Cóme, D., *J. Exp. Bot.* 2001, *52*, 701–708.

[58] Pammenter, N. W., Berjak, P., *Seed Sci. Res.* 1999, *9*, 13–37.

[59] Zhu, D., Scandalios, J. G., *Natl. Acad. Sci.* 1993, *90*, 9310–9314.

[60] Pukacka, S., Ratajczak, E., *J. Plant Physiol.* 2006, *163*, 1259–1266.

[61] Wojtyla, L., Garnczarska, M., Zalewski, T., Bednarski, W., Ratajczak, L., Jurga, S., *J. Plant Physiol.* 2006, *163*, 1207–1220.

[62] Ravet, K., Touraine, B., Boucherez, J., Briat, J. F., Gaymard, F., Cellier, F., *Plant J.* 2009, *57*, 400–412.

[63] Castillo, J., Genoves, A., Franco, L., Rodrigo, M. I., *J. Exp. Bot.* 2005, *56*, 3159–3169.

[64] Chiatante, D., Onelli, E., Patrignani, G., Scippa, G. S., *J. Exp. Bot.* 1995, *6*, 815–821.

[65] Castillo, J., Rodrigo, M. I., Marquez, J. A., Zuniga, A., Franco, L., *Eur. J. Biochem.* 2000, *267*, 2156–2165.

[66] Battaglia, M., Olvera-Carrillo, Y., Garciarrubio, A., Campos, F., Covarrubias, A. A., *Plant Physiol.* 2008, *148*, 6–24.

[67] Lechner, E., Achard, P., Vansiri, A., Potuschak, T., Genschik, P., *Curr. Opin. Plant Biol.* 2006, *9*, 631–638.

[68] Sharon, N., Lis, H., *FASEB J.* 1990, *4*, 3198–3208.

[69] Roopashree, S., Singh, S. A., Gowda, L. R., Rao, A. G. A., *Biochem. J.* 2006, *395*, 629–639.

[70] de Azevedo Pereira, R., Valencia-Jiménez, A., Magalhães, C. P., Prates, M. V., Melo, J. A. T., de Lima, L. M., de Sales, M. P. *et al.*, *J. Agric. Food Chem.* 2007, *55*, 10714–10719.

[71] Qi, R. F., Song, Z. W., Chi, C. W., *Acta Biochim. Biophys. Sin.* 2005, *37*, 283–292.

[72] Kennedy, A. R., *Pharmacol. Ther.* 1998, *78*, 167–209.

[73] Buckeridge, M. S., Reid, J. S., *Planta* 1994, *192*, 502–511.

[74] Sekimata, M., Ogura, K., Tsumuraya, Y., Hasimoto, Y., Yamamoto, S.,*Plant Physiol.* 1989, *96*, 282–292.

[75] Barróco, R. M., Van Poucke, K., Bergervoet, J. H. W., De Veylder, L., Groot, S. P. C., Inzé, D., Engler, G., *Plant Physiol.* 2005, *137*, 127–140.

[76] Walz, A., Park, S., Slovin, J. P., Ludwig-Müller, J., Momonoki, Y. S., Cohen, J. D., *Proc. Natl. Acad. Sci.* USA 2002, *99*, 1718–1723.

[77] Mockaitis, K., Estelle, M., *Annu. Rev. Cell Dev. Biol.* 2008, *24*, 55–80.

[78] Echeverrigaray, S., Oliveira, A. C., Carvalho, M. T. V., Derbyshire, E., *J. Genetic Breed.* 1998, *52*, 89–94.

[79] Piergiovanni, A. R., Taranto, G., *Plant Genet. Resour. Newsl.* 2005, *141*, 42–47.

[80] Yüzbaşioğlu, E., Açik, L., Özcan, S., *Biol. Plant* 2008, *52*, 126–128.

SUPPORTING INFORMATION

Statistical analysis

A correlation matrix was computed on the basis of proteome quantitative data. This is a symmetrical matrix that describes correlation among variables and it was used to perform multivariate statistical analysis. Principal Component Analysis (PCA), a procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables (PCs), was performed to synthetize proteomic variability in few linear combinations (PCs), containing the information of all correlated original variables (proteins). Then, a confidential ellipse was computed on Capracotta populations to test the significance of grouping. In particular, it indicated the statistical range with a specified probability (95%) that a given specimens lies within the group of Capracotta. Moreover, we used PC coefficients to detect the more weighted proteins, in the explained pattern of proteome variability. In fact, these coefficients indicated the weight of a protein along a principal component. Finally, Neighbor-Joining (NJ) method was used for the construction of phylogenetic trees. This method clusters together alike pair within a group of related objects to create a tree whose branches reflect the degree of difference among the objects. To test the significance of the nodes derived from NJ analysis, bootstrapping was computed at 999 permutations. In detail, the bootstrap value of a node represents an estimation of the statistical significance of a particular branching point in a tree, it is the percentage of times that a node is present in the set of trees that is constructed from the data sets.

Table S1. Proteins identified in the 2-DE proteomic map of *L. culinaris* mature seeds. Spot number, accession number, protein name, experimental and theoretical pI and Mr values, method of identification, i.e. MALDI-TOF peptide mass fingerprint (PMF) or nanoLC-ESI-LIT-MS/MS (LCMS), number of peptides identified, sequence coverage, identification score, organism, and function are listed. Spot numbering refers to Figure 1. [(C)] indicates constantly expressed proteins. MASCOT (labeled with asterisk) and Sequest scores were reported for PMF or LCMS analysis, respectively.

Spot	Accession	Description	Theoretical Mr (Kda)/pI	Experimental Mr (Kda)/pI	MS	Peptides	Coverage	Score	Species	Functional Class
1	gi137582	vicilin	52.23 / 5.39	9.89 / 5.22	LCMS	2	5	7.17	*P. sativum*	protein destination and storage
	gi1297070	convicilin	62.81 / 6.09		LCMS	2	5	6.01	*V. narbonensis*	protein destination and storage
2	gi186513260	ATP-DNA binding DNA directed polymerase	93.83 / 7.84	10.81 / 5.16	PMF	17	21	71*	*A. thaliana*	cell growth/division
3	gi29539109	allergen Len c 1.0101	47.83 / 5.35	14.42 / 5.58	PMF	7	16	74*	*L. culinaris*	protein destination and storage
4	gi137581	provicilin, type B	46.39 / 5.39	16.12 / 5.81	LCMS	2	5	5.23	*P. sativum*	protein destination and storage
5	gi29539109	allergen Len c 1.0101	47.83 / 5.35	16.10 / 5.45	LCMS	6	13	24.89	*L. culinaris*	protein destination and storage
	gi42414629	vicilin	47.30 / 5.43		LCMS	3	14	16.54	*P. sativum*	protein destination and storage
6	gi75243366	allergen Len c 1.0101	47.83 / 5.35	15.30 / 5.47	PMF	9	18	105*	*L. culinaris*	protein destination and storage
7	gi758248	vicilin precursor	49.52 / 5.33	18.27 / 5.16	LCMS	4	8	43.36	*P. sativum*	protein destination and storage
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		LCMS	4	8	40.62	*L. culinaris*	protein destination and storage
	gi87162566	cupin, RmlC-type	53.15 / 5.42		LCMS	4	8	34.88	*M. truncatula*	protein destination and storage
	gi110611256	lectin	29.52 / 5.10		LCMS	2	8	5.97	*C. arietinum*	disease/defence
8	gi29539111	allergen Len c 1.0102	47.47 / 5.34	16.55 / 5.22	LCMS	2	6	16.08	*L. culinaris*	protein destination and storage

	gi137581	provicilin, type B	46.39 / 5.39		LCMS	2	3	9.26	*P. sativum*	protein destination and storage
9	gi224144326	ATP-binding DNA ligase 4	139.32 / 6.93	16.60 / 6.21	PMF	12	14	86*	*P. trichocarpa*	cell growth/division
10	gi225043	legumin A	26.97 / 9.32	22.50 / 7.85	LCMS	6	14	30.56	*V. faba*	protein destination and storage
	gi479104	legumin B	54.31 / 6.23		LCMS	5	8	21.26	*V. sativa*	protein destination and storage
11	gi75331682	lectin, beta chain	30.28 / 5.37	18.48 / 7.40	LCMS	4	22	17.80	*L. culinaris*	disease/defence
12	gi29539109	allergen Len c 1.0101	47.83 / 5.35	19.25 / 7.16	PMF	10	24	73*	*L. culinaris*	protein destination and storage
13	gi553082	legumin precursor	23.42 / 6.75	9.84 / 6.01	LCMS	2	6	13.13	*P. sativum*	protein destination and storage
14	gi29539109	allergen Len c 1.0101	47.83 / 5.35	14.23 / 5.85	LCMS	2	6	12.31	*L. culinaris*	protein destination and storage
15	gi81954	vicilin precursor	49.52 / 5.33	15.63 / 6.00	PMF	7	16	73*	*P. sativum*	protein destination and storage
16	gi1754979	Em protein	10.81 / 5.75	15.49 / 6.07	LCMS	2	25	12.31	*R. pseudoacacia*	disease/defence
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		LCMS	2	5	8.30	*L. culinaris*	protein destination and storage
17	gi75184070	vicilin 47k	17.82 / 9.24	21.30 / 6.45	PMF	6	28	68*	*P. sativum*	protein destination and storage
18	gi29539111	allergen Len c 1.0102	47.47 / 5.34	20.08 / 5.88	LCMS	5	10	38.42	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	5	9	37.73	*L. culinaris*	protein destination and storage
	gi87162566	cupin, RmlC-type	53.15 / 5.42		LCMS	4	7	34.01	*M. truncatula*	protein destination and storage
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	4	7	32.72	*P. sativum*	protein destination and storage
19	gi758248	vicilin precursor	49.52 / 5.33	25.32 / 6.01	LCMS	3	5	20.51	*P. sativum*	protein destination and storage
	gi479104	legumin B	54.35 / 6.23		LCMS	2	6	14.48	*V. sativa*	protein destination and storage
	gi282925	legumin K	56.28 / 5.65		LCMS	2	9	11.97	*P. sativum*	protein destination and storage
20	gi29539111	allergen Len c 1.0102	47.47 / 5.34	22.56 / 6.14	LCMS	2	5	9.01	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	3	7	14.24	*L. culinaris*	protein destination and storage

	gi137582	vicilin	52.23 / 5.39		LCMS	4	9	20.21	*P. sativum*	protein destination and storage
	gi137581	provicilin	46.39 / 5.39		LCMS	2	5	12.84	*P. sativum*	protein destination and storage
21	gi29539111	allergen Len c 1.0102	47.47 / 5.34	21.00 / 5.80	PMF	17	31	186*	*L. culinaris*	protein destination and storage
22	gi75184070	vicilin 47k	17.82 / 9.24	20.78 / 5.71	PMF	5	30	76*	*P. sativum*	protein destination and storage
23	gi75184070	vicilin 47k	17.82 / 9.24	20.95 / 5.44	PMF	6	30	83*	*P. sativum*	protein destination and storage
24	gi122182474	cupin, RmlC-type	56.08 / 5.99	19.83 / 6.09	PMF	12	22	108*	*M. truncatula*	protein destination and storage
25	gi29539109	allergen Len c 1.0101	47.83 / 5.35	25.63 / 6.25	LCMS	5	8	12.18	*L. culinaris*	protein destination and storage
26	gi2765097	P54 protein	54.66 / 6.05	25.42 / 6.18	PMF	21	37	129*	*P. sativum*	disease/defence
27	gi29539109	allergen Len c 1.0101	47.83 / 5.35	26.97 / 6.46	LCMS	3	8	10.19	*L. culinaris*	protein destination and storage
	gi2765097	P54 protein	54.66 / 6.05		PMF	23	40	163*	*P. sativum*	disease/defence
28	gi2765097	P54 protein	54.66 / 6.05	27.91 / 6.40	PMF	18	36	143*	*P. sativum*	disease/defence
	gi33285914	putative dehydroascorbate reductase	12.04 / 6.15		LCMS	2	15	15.30	*B. rapa*	disease/ defence
29	gi113570	albumin-2 (PA2)	26.24 / 5.16	29.50 / 6.35	PMF	15	5	80*	*P. sativum*	protein destination and storage
30	gi137582	vicilin	52.23 / 5.39	31.67 / 6.33	LCMS	3	7	16.70	*P. sativum*	protein destination and storage
31	gi758248	vicilin precursor	49.52 / 5.33	33.19 / 6.33	LCMS	10	19	70.65	*P. sativum*	protein destination and storage
32	gi758248	vicilin precursor	49.52 / 5.33	31.71 / 6.55	LCMS	7	12	50.01	*P. sativum*	protein destination and storage
33	gi758248	vicilin precursor	49.52 / 5.33	31.36 / 6.60	LCMS	3	8	15.97	*P. sativum*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	3	8	15.80	*L. culinaris*	protein destination and storage
	gi1297070	convicilin precursor	62.81 / 6.09		LCMS	2	4	6.26	*V. narbonensis*	protein destination and storage
34	gi113570	albumin-2 (PA2)	26.24 / 5.16	29.83 / 6.81	PMF	8	21	71*	*P. sativum*	protein destination and storage
35	gi7488185	probable serine proteinase	25.39 / 5.50	27.98 / 7.37	PMF	4	23	65*	*A. thaliana*	protein destination and storage

36	gi14488168	lectin	27.89 / 5.34	31.96 / 5.56	PMF	5	22	77*	*L. culinaris*	disease/defence
37	gi137579	provicilin, type A	31.54 / 5.57	30.08 / 5.06	LCMS	3	20	24.46	*P. sativum*	protein destination and storage
	gi217075454	nascent polypeptide associated complex NAC	22.13 / 4.44		LCMS	2	13	10.78	*M. truncatula*	protein destination and storage
39	gi15241107	seed maturation family protein	19.52 / 4.89	35.49 / 5.06	LCMS	2	6	13.95	*A. thaliana*	disease/defence
	gi38232568	translational elongation factor 1 subunit beta	25.31 / 4.41		LCMS	2	8	12.34	*P. sativum*	protein synthesis
	gi137579	provicilin, type A	31.54 / 5.57		LCMS	2	16	20.02	*P. sativum*	protein destination and storage
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	2	6	8.97	*P. sativum*	protein destination and storage
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		LCMS	2	5	4.50	*L. culinaris*	protein destination and storage
40	gi1052778	ferritin	28.65 / 6.01	29.41 / 6.07	PMF	5	21	77*	*P. sativum*	protein destination and storage
41	gi113570	albumin-2 (PA2)	26.24 / 5.16	30.87 / 6.01	PMF	16	6	96*	*P. sativum*	protein destination and storage
42	gi758248	vicilin precursor	49.52 / 5.33	31.56 / 5.98	LCMS	4	12	28.51	*P. sativum*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	4	12	26.39	*L. culinaris*	protein destination and storage
	gi1297072	vicilin precursor	52.41 / 5.52		LCMS	4	11	26.15	*V. narbonensis*	protein destination and storage
	gi137579	provicilin, type A	31.54 / 5.57		LCMS	3	20	25.03	*P. sativum*	protein destination and storage
	gi82173888	provicilin precursor	51.42 / 6.04		LCMS	4	7	23.99	*C. arietinum*	protein destination and storage
	gi112744	2S seed storage protein	34.07 / 8.74		LCMS	2	8	8.54	*H. annuus*	protein destination and storage
43	gi29539111	allergen Len c 1.0102	47.47 / 5.34	33.94 / 5.96	LCMS	8	17	61.00	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	8	17	60.49	*L. culinaris*	protein destination and storage
	gi137582	vicilin	52.23 / 5.39		LCMS	7	16	52.47	*P. sativum*	protein destination and storage

	gi137584	vicilin	52.69 / 5.77		LCMS	5	10	27.11	*V. faba*	protein destination and storage
	gi137581	provicilin	46.39 / 5.39		LCMS	5	9	25.77	*P. sativum*	protein destination and storage
	gi82173888	provicilin	51.42 / 6.04		LCMS	4	9	21.59	*C. arietinum*	protein destination and storage
44	gi137582	vicilin precursor	52.23 / 5.39	33.77 / 5.82	LCMS	2	7	9.01	*P. sativum*	protein destination and storage
45	gi29539109	allergen Len c 1.0101	47.83 / 5.35	35.26 / 5.82	PMF+LCMS	10+5	26	108*	*L. culinaris*	protein destination and storage
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		PMF+LCMS	10+7	29	107*	*L. culinaris*	protein destination and storage
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	5	13	30.07	*P. sativum*	protein destination and storage
46	gi7442027	legumin A	57.62 / 6.11	38.74 / 5.81	LCMS	2	4	10.10	*P. sativum*	protein destination and storage
	gi600108	legumin A	54.67 / 7.00		LCMS	3	9	14.77	*V. narbonensis*	protein destination and storage
	gi126161	legumin A2	59.27 / 6.21		LCMS	4	7	20.55	*P. sativum*	protein destination and storage
47	gi7442027	legumin A	57.62 / 6.11	38.93 / 5.68	LCMS	3	4	18.69	*P. sativum*	protein destination and storage
	gi600108	legumin A	54.67 / 7.00		LCMS	5	12	26.84	*V. narbonensis*	protein destination and storage
	gi126161	legumin A2	59.27 / 6.21		LCMS	5	7	26.61	*P. sativum*	protein destination and storage
	gi2765097	P54 protein	54.66 / 6.05		LCMS	2	7	8.41	*P. sativum*	disease/defence
48	gi7442027	legumin A	57.62 / 6.11	38.98 / 5.55	LCMS	3	4	15.49	*P. sativum*	protein destination and storage
	gi126161	legumin A2	59.27 / 6.21		LCMS	5	7	25.91	*P. sativum*	protein destination and storage
49	gi2765097	P54 protein	54.66 / 6.05	40.78 / 5.63	LCMS	5	14	30.26	*P. sativum*	disease/defence
	gi7442027	legumin A	57.62 / 6.11		LCMS	2	4	9.84	*P. sativum*	protein destination and storage
	gi126161	legumin A2	59.27 / 6.21		LCMS	3	6	12.79	*P. sativum*	protein destination and storage
50	gi126170	legumin J	56.90 / 5.70	42.78 / 5.53	LCMS	4	17	23.88	*P. sativum*	protein destination and storage
	gi542002	legumin, type B	32.73 / 5.23		LCMS	2	10	11.41	*V. faba*	protein destination and storage

	gi282925	legumin K	56.28 / 5.65		LCMS	5	18	32.48	*P. sativum*	protein destination and storage
51	gi126170	legumin J	56.90 / 5.70	42.71 / 5.46	LCMS	5	17	36.50	*P. sativum*	protein destination and storage
	gi542002	legumin, type B	32.73 / 5.23		LCMS	2	10	10.01	*V. faba*	protein destination and storage
	gi282925	legumin K	56.28 / 5.65		LCMS	5	18	33.46	*P. sativum*	protein destination and storage
	gi479104	legumin B	54.35 / 6.23		LCMS	2	7	10.04	*V. sativa*	protein destination and storage
52	gi126170	legumin J	56.90 / 5.70	42.92 / 5.39	LCMS	2	4	12.57	*P. sativum*	protein destination and storage
	gi542002	legumin, type B	32.73 / 5.23		LCMS	2	10	10.43	*V. faba*	protein destination and storage
53	gi542002	legumin, type B	32.73 / 5.23	43.03 / 5.33	LCMS	2	10	9.94	*V. faba*	protein destination and storage
54	gi7688242	convicilin	60.13 / 5.56	68.10 / 5.10	PMF	15	24	158*	*L. culinaris*	protein destination and storage
55	gi758248	vicilin precursor	49.52 / 5.33	40.66 / 5.93	LCMS	2	6	21.58	*P. sativum*	protein destination and storage
	gi137579	provicilin, type A	31.54 / 5.57		LCMS	2	16	17.64	*P. sativum*	protein destination and storage
	gi87162566	cupin, RmlC-type	53.15 / 5.42		LCMS	2	5	11.94	*M. truncatula*	protein destination and storage
	gi259474	legumin propolypeptide alpha chain	31.96 / 5.11		LCMS	3	21	19.00	*Papilionoideae*	protein destination and storage
	gi126171	legumin K alpha and beta chain	39.78 / 5.38		LCMS	2	3	10.55	*P. sativum*	protein destination and storage
56	gi29539109	allergen Len c 1.0101	47.83 / 5.35	37.69 / 5.96	PMF	12	27	123*	*L. culinaris*	protein destination and storage
57	gi29539109	allergen Len c 1.0101	47.83 / 5.35	38.21 / 6.06	PMF	11	27	87*	*L. culinaris*	protein destination and storage
58	gi75243366	allergen Len c 1.0101	47.83 / 5.35	38.12 / 6.18	PMF	18	41	225*	*L. culinaris*	protein destination and storage
59	gi29539109	allergen Len c 1.0101	47.83 / 5.35	38.29 / 6.34	PMF	11	27	101*	*L. culinaris*	protein destination and storage
60	gi29539109	allergen Len c 1.0101	47.83 / 5.35	37.67 / 6.74	LCMS	2	5	11.02	*L. culinaris*	protein destination and storage
61	gi29539109	allergen Len c 1.0101	47.83 / 5.35	37.88 / 6.84	PMF	9	22	93*	*L. culinaris*	protein destination

										and storage
62	gi57339044	catalase	54.94 / 6.59	41.02 / 7.14	PMF	9	25	102*	*S. tuberosum*	disease/defence
63	gi27805509	maturase K	60.28 / 9.66	54.25 / 7.41	PMF	12	26	71*	*A. petiolata*	transcription
64	gi27805509	maturase K	60.28 / 9.66	53.23 / 7.31	PMF	12	26	71*	*A. petiolata*	transcription
65	gi27805509	maturase K	60.28 / 9.66	52.87 / 7.20	PMF	12	26	71*	*A. petiolata*	transcription
67	gi7688242	convicilin	60.13 / 5.56	69.84 / 7.12	PMF	8	16	80*	*L. culinaris*	protein destination and storage
68	gi7688242	convicilin	60.13 / 5.56	69.65 / 6.99	PMF	10	17	91*	*L. culinaris*	protein destination and storage
70	gi2765097	P54 protein	54.66 / 6.05	48.33 / 6.74	PMF	10	15	73*	*P. sativum*	disease/ defence
72	gi758248	vicilin precursor	49.52 / 5.33	47.93 / 6.55	LCMS	5	13	30.07	*P. sativum*	protein destination and storage
74	gi758248	vicilin precursor	49.52 / 5.33	51.60 / 6.40	LCMS	5	13	31.14	*P. sativum*	protein destination and storage
75	gi758248	vicilin precursor	49.52 / 5.33	55.60 / 6.30	LCMS	5	13	33.04	*P. sativum*	protein destination and storage
76	gi758248	vicilin precursor	49.52 / 5.33	55.61 / 6.24	PMF+LCMS	9+2	24	116*	*P. sativum*	protein destination and storage
77	gi758248	vicilin precursor	49.52 / 5.33	55.21/ 6.20	PMF	8	24	93*	*P. sativum*	protein destination and storage
78	gi758248	vicilin precursor	49.52 / 5.33	58.88 / 6.22	PMF	8	23	104*	*P. sativum*	protein destination and storage
79	gi75184075	convicilin	60.13 / 5.56	68.75 / 6.24	PMF	12	30	137*	*L. culinaris*	protein destination and storage
80	gi75184075	convicilin	60.13 / 5.56	69.55 / 6.16	PMF	10	21	119*	*L. culinaris*	protein destination and storage
81	gi42521309	enolase	47.72 / 5.31	64.73 / 6.08	PMF	14	37	96*	*G. max*	energy
83	gi29539111	allergen Len c 1.0102	47.47 / 5.34	50.00 / 6.00	LCMS	7	16	55.89	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	7	16	54.91	*L. culinaris*	protein destination and storage
	gi137582	vicilin	52.23 / 5.39		LCMS	8	18	60.28	*P. sativum*	protein destination and storage
	gi137584	vicilin	52.69 / 5.77		LCMS	4	7	23.44	*V. faba*	protein destination and storage
	gi137581	provicilin	46.39 / 5.39		LCMS	4	9	20.11	*P. sativum*	protein destination and storage
	gi82173888	provicilin	51.42 / 6.04		LCMS	5	11	22.48	*C. arietinum*	protein destination and storage

	gi2765097	P54 protein	54.66 / 6.05		LCMS	2	5	7.48	*P. sativum*	disease/defence
84	gi29539111	allergen Len c 1.0102	47.47 / 5.34	50.41 / 5.92	LCMS	7	16	54.26	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	7	16	53.04	*L. culinaris*	protein destination and storage
	gi137582	vicilin	52.23 / 5.39		LCMS	8	18	61.43	*P. sativum*	protein destination and storage
	gi137584	vicilin	52.69 / 5.77		LCMS	4	7	20.94	*V. faba*	protein destination and storage
	gi137581	provicilin	46.39 / 5.39		LCMS	4	9	21.03	*P. sativum*	protein destination and storage
	gi82173888	provicilin	51.42 / 6.04		LCMS	5	11	24.04	*C. arietinum*	protein destination and storage
	gi2765097	P54 protein	54.66 / 6.05		LCMS	6	15	51.30	*P. sativum*	disease/defence
85	gi2765097	P54 protein	54.66 / 6.05	49.53 / 5.82	LCMS	6	15	50.49	*P. sativum*	disease/defence
86	gi225447450	glucose-1-P adenyltransferase small subunit	55.90 / 6.53	56.76 / 5.83	PMF	12	30	85*	*V. vinifera*	energy
87	gi2765097	P54 protein	54.66 / 6.05	49.60 / 5.70	LCMS	6	15	51.44	*P. sativum*	disease/defence
88	gi2765097	P54 protein	54.66 / 6.05	49.77 / 5.57	LCMS	6	15	52.01	*P. sativum*	disease/defence
89	gi2765097	P54 protein	54.66 / 6.05	49.88 / 5.53	LCMS	6	15	51.30	*P. sativum*	disease/defence
90	gi2116558	F1 ATPase	60.15 / 6.63	67.24 / 5.66	PMF	13	23	129*	*P. sativum*	energy
91	gi7688242	convicilin	60.13 / 5.56	68.13 / 5.39	PMF	9	17	101*	*L. culinaris*	protein destination and storage
92	gi122189405	cyclin-like F-box	45.38 / 9.10	20.20 / 6.42	PMF	8	25	69*	*M. truncatula*	cell growth/division
94	gi562006	PsHSP71.2	71.17 / 5.17	102.17 / 5.52	PMF	10	19	91*	*P. sativum*	disease/defence
96	gi29539111	allergen Len c 1.0102	47.47 / 5.34	24.23 / 5.77	LCMS	2	5	11.36	*L. culinaris*	protein destination and storage
	gi600108	legumin A	54.67 / 7.00		LCMS	2	4	9.08	*V. narbonensis*	protein destination and storage
97	gi7688242	convicilin	60.13 / 5.56	15.22 / 7.80	LCMS	6	8	54.37	*L. culinaris*	protein destination and storage
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		LCMS	5	14	40.08	*L. culinaris*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	5	14	39.11	*L. culinaris*	protein destination and storage
	gi137582	vicilin	52.23 / 5.39		LCMS	4	11	23.07	*P. sativum*	protein destination and storage

	gi137581	provicilin	46.39 / 5.39		LCMS	2	5	11.45	*P. sativum*	protein destination and storage
98	gi159163726	trypsin/chymotrypsin Bowman-Birk Inhibitor	7.46 / 5.84	8.85 / 7.65	PMF	4	67	71*	*L. culinaris*	disease/defence
99	gi2765097	P54 protein	54.66 / 6.05	43.90 / 6.23	LCMS	5	11	12.88	*P. sativum*	disease/defence
100	gi124360836	aldo/keto reductase	37.40 / 5.77	43.74 / 6.17	PMF	12	32	75*	*M. truncatula*	disease/defence
101	gi137582	vicillin	52.23 / 5.39	33.44 / 6.15	PMF	12	33	71*	*P. sativum*	protein destination and storage
102	gi137582	vicillin	52.23 / 5.39	33.56 / 6.21	PMF	12	33	70*	*P. sativum*	protein destination and storage
104(C)	gi29539109	allergen Len c 1.0101	47.83 / 5.35	16.09 / 5.34	LCMS	3	8	22.14	*L. culinaris*	protein destination and storage
	gi42414629	vicilin	47.39 / 5.43		LCMS	3	8	21.73	*P. sativum*	protein destination and storage
	gi137579	provicilin	31.54 / 5.57		LCMS	2	8	9.93	*P. sativum*	protein destination and storage
105(C)	gi29539109	allergen Len c 1.0101	47.83 / 5.35	15.34 / 5.71	PMF	13	22	148*	*L. culinaris*	protein destination and storage
106(C)	gi1297070	convicilin precursor	62.81 / 6.09	10.20 / 5.60	LCMS	2	6	13.66	*V. narbonensis*	protein destination and storage
107(C)	gi29539111	allergen Len c 1.0102	47.47 / 5.34	12.51 / 5.35	LCMS	3	8	10.08	*L. culinaris*	protein destination and storage
	gi42414629	vicilin	47.30 / 5.43		LCMS	2	6	6.93	*P. sativum*	protein destination and storage
108(C)	gi29539111	allergen Len c 1.0102	47.47 / 5.34	14.20 / 7.05	PMF	10	20	117*	*L. culinaris*	protein destination and storage
109(C)	gi29539111	allergen Len c 1.0102	47.47 / 5.34	20.93 / 5.56	PMF	16	32	202*	*L. culinaris*	protein destination and storage
110(C)	gi29539111	allergen Len c 1.0102	47.47 / 5.34	19.59 / 5.33	PMF	11	26	139*	*L. culinaris*	protein destination and storage
111(C)	gi29539109	allergen Len c 1.0101	47.83 / 5.35	20.72 / 5.99	PMF	9	18	82*	*L. culinaris*	protein destination and storage
112(C)	gi7339555	vicilin 47k precursor	17.82 / 9.24	20.43 / 6.19	LCMS	4	22	28.78	*P. sativum*	protein destination and storage
113(C)	gi29539109	allergen Len c 1.0101	47.83 / 5.35	19.74 / 6.68	PMF	11	25	110*	*L. culinaris*	protein destination and storage
114(C)	gi110611256	lectin	29.52 / 5.10	18.29 / 6.80	LCMS	2	8	10.86	*C. arietinum*	disease/defence
115(C)	gi29539111	allergen Len c 1.0102	47.47 / 5.34	19.76 / 6.91	LCMS	2	5	19.99	*L. culinaris*	protein destination and storage

116(C)	gi62910855	lectin	30.35 / 5.24	18.24 / 6.91	PMF+LCMS	6+1	19	80*	*L. culinaris*	disease/defence
117(C)	gi11967889	Mn-superoxide dismutase 2	9.35 / 6.79	25.73 / 6.75	LCMS	2	20	10.46	*P. persica*	disease/defence
118(C)	gi479104	legumin B	54.35 / 6.23	24.07 / 6.24	LCMS	2	6	22.71	*V. sativa*	protein destination and storage
	gi7339555	vicilin 47k precursor	17.82 / 9.24		LCMS	2	14	9.54	*P. sativum*	protein destination and storage
119(C)	gi7339555	vicilin 47k precursor	17.82 / 9.24	24.40 / 6.18	LCMS	2	7	12.55	*P. sativum*	protein destination and storage
120(C)	gi219981096	protein product beta-galactosidase-like	87.42 / 9.28	27.70 / 5.24	LCMS	2	2	13.53	*A. thaliana*	metabolism
121(C)	gi600108	legumin A	54.67 / 7.00	39.03 / 5.91	LCMS	2	6	36.17	*V. narbonensis*	protein destination and storage
	gi6273402	legumin, alpha and beta subunit	56.25 / 6.20		LCMS	2	5	29.79	*C. arietinum*	protein destination and storage
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	4	8	23.97	*L. culinaris*	protein destination and storage
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	4	8	17.22	*P. sativum*	protein destination and storage
	gi2765097	P54 protein	54.66 / 6.05		LCMS	3	5	15.05	*P. sativum*	disease/defence
122(C)	gi7688242	convicilin	60.13 / 5.56	40.45 / 7.24	LCMS	6	10	28.35	*L. culinaris*	protein destination and storage
123(C)	gi26000394	putative TAG-associated factor	32.21 / 6.33	37.84 / 7.04	LCMS	3	10	17.40	*L. angustifolius*	metabolism
	gi29539111	allergen Len c 1.0102	47.47 / 5.34		LCMS	2	5	11.32	*L. culinaris*	protein destination and storage
125(C)	gi29539109	allergen Len c 1.0101	47.83 / 5.35	18.43 / 7.27	PMF	10	20	98*	*L. culinaris*	protein destination and storage
126(C)	gi195629288	16.9 kDa class I heat shock protein 1	16.98 / 6.77	18.46 / 7.48	LCMS	2	12	17.57	*Z. mays*	disease/defence
	gi29539109	allergen Len c 1.0101	47.83 / 5.35		LCMS	3	8	13.83	*L. culinaris*	protein destination and storage
	gi110611256	lectin	29.52 / 5.10		LCMS	2	8	5.67	*C. arietinum*	disease/defence
129(C)	gi758248	vicilin precursor	49.52 / 5.33	57.54 / 6.40	PMF+LCMS	12+6	30	100*	*P. sativum*	protein destination and storage
	gi7688242	convicilin	60.13 / 5.56		LCMS	4	8	17.09	*L. culinaris*	protein destination and storage
	gi224143515	similar to MYB transcription factor	41.14 / 8.17		LCMS	2	3	9.17	*P. trichocarpa*	transcription

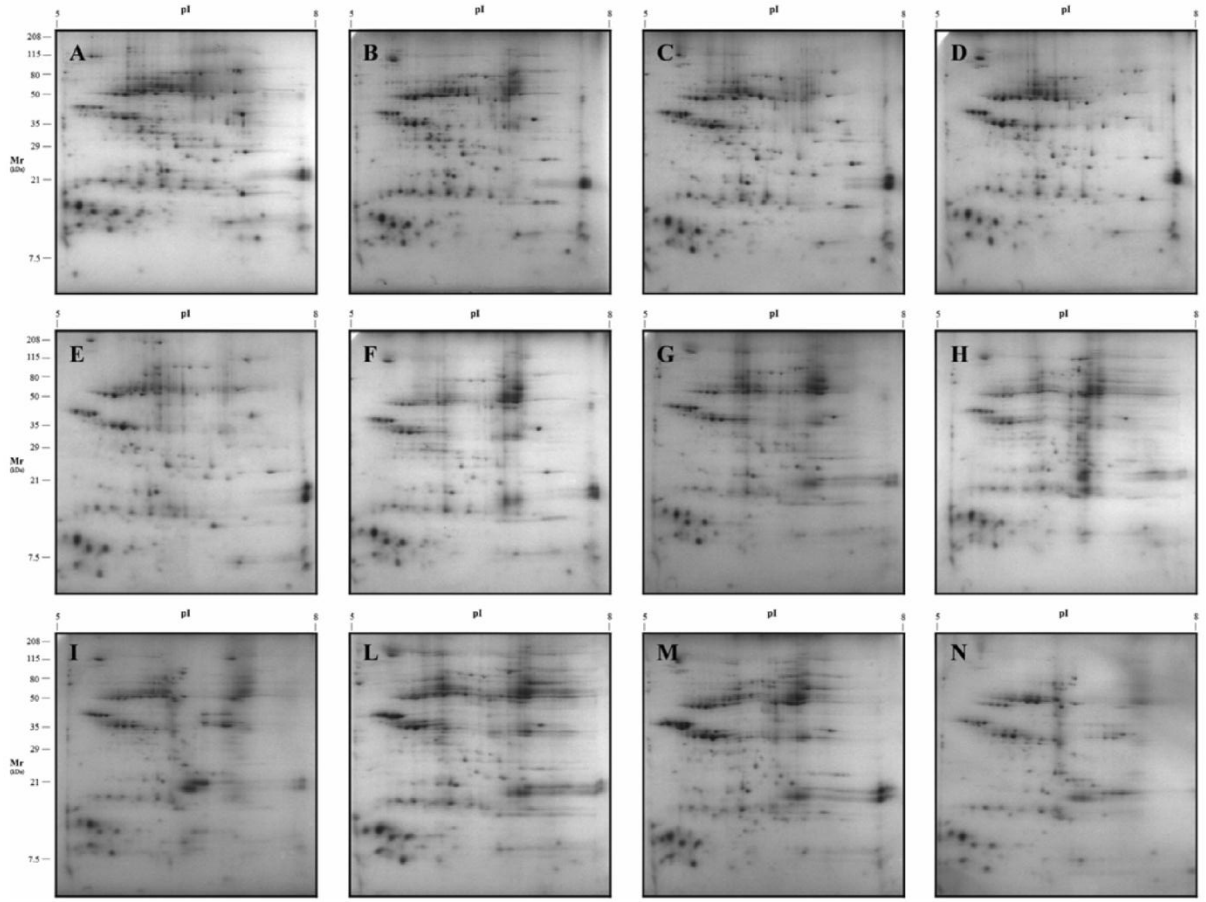
130(C)	gi758248	vicilin precursor	49.52 / 5.33	57.00 / 6.51	LCMS	4	8	41.85	*P. sativum*	protein destination and storage
	gi7688242	convicilin	60.13 / 5.56		LCMS	2	4	5.12	*L. culinaris*	protein destination and storage
131(C)	gi26000758	IAP1	35.52 / 6.20	51.30 / 6.90	LCMS	2	4	22.06	*P. vulgaris*	others
132(C)	gi26000758	IAP1	35.52 / 6.20	52.30 / 6.96	LCMS	2	4	36.72	*P. vulgaris*	others
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	3	8	15.79	*P. sativum*	protein destination and storage
133(C)	gi26000758	IAP1	35.52 / 6.20	52.22 / 7.08	LCMS	2	4	27.18	*P. vulgaris*	others
	gi758248	vicilin precursor	49.52 / 5.33		LCMS	3	8	12.67	*P. sativum*	protein destination and storage
134(C)	gi758248	vicilin precursor	49.52 / 5.33	33.52 / 6.08	LCMS	7	16	42.23	*P. sativum*	protein destination and storage
	gi169927	beta conglycinin alpha subunit	24.45 / 5.43		LCMS	2	6	16.37	*G. max*	protein destination and storage
135(C)	gi479104	legumin B	54.35 / 6.23	24.4/7.85	LCMS	10	9	60.88	*V. sativa*	protein destination and storage
	gi225043	legumin A	26.97 / 9.32		LCMS	7	15	51.66	*V. faba*	protein destination and storage
	gi600108	legumin A	54.67 / 7.00		LCMS	3	5	38.60	*V. narbonensis*	protein destination and storage
	gi126171	legumin K alpha and beta chain	39.78 / 5.38		LCMS	4	6	34.07	*P. sativum*	protein destination and storage

Principal Component 1			Principal Component 2		
Ref. Map ID	Coeff.	Corr.	Ref. Map ID	Coeff.	Corr.
6	-0.102	-0.600	5	0.176	0.563
10	-0.747	-0.935	10	-0.364	-0.302
15	-0.109	-0.849	46	-0.260	-0.579
20	-0.103	-0.701	50	-0.198	-0.503
35	-0.118	-0.701	51	-0.500	-0.769
49	0.109	0.707	52	-0.176	-0.413
50	-0.108	-0.414	53	-0.143	-0.362
51	0.231	0.537	56	-0.424	-0.632
52	0.134	0.473	57	-0.128	-0.380
53	0.208	0.792	63	-0.155	-0.597
56	0.183	0.411	65	-0.100	-0.542
77	0.124	0.657	68	-0.101	-0.668
92	-0.104	-0.705	83	-0.166	-0.559
97	-0.180	-0.746	85	-0.143	-0.527
98	-0.104	-0.742	87	-0.104	-0.569

Supporting Information Table S2. Structure matrix extracted from the PCA performed on 103 variable proteins. Coefficients and PC correlations were showed for the 24 proteins that resulted more indicative in PCA for discriminating the different landraces.

PC COEFFICIENTS							
Ref. Map ID	PC1	PC2	PC3	Ref. Map ID	PC1	PC2	PC3
2	-	0.118	-	**59**	-	-0.299	
3	-	-	-0.118	**64**	0.112	-	-
5	-	0.186	-	**65**	-	-	-0.125
6	-0.119	-	-	**66**	-	-	0.317
7	0.107	-	-0.164	**67**	-	-	-0.188
10	-0.778	0.248	0.132	**71**	-	-	-0.176
14	-	-	-0.104	**72**	-	-0.098	0.153
17	-0.118	-	-0.306	**74**	-	-0.157	-
20	-	-	-0.247	**75**	-	-	-0.108
22	-	-	-0.101	**78**	-	-0.392	-0.153
29	-	-	-0.173	**80**	-	-0.237	-
30	-	-	-0.098	**81**	-	-	0.150
34	-	-	-0.118	**82**	-0.126	-	-0.117
35	-0.125	0.163	-0.147	**83**	-0.141	-0.118	-
46	-0.153	-0.415	0.139	**84**	-0.100	-0.159	-
47	-0.142	-0.162	0.147	**85**	-	-	-0.158
50	-0.139	-0.219	-0.118	**87**	-	-	-0.159
51	-	-0.164	-	**92**	-0.101	-	-0.231
52	-	-0.127	-	**93**	-	-0.098	0.153
53	-	-	0.153	**97**	-0.223	-0.167	-
57	0.133	-	-0.240	**98**	-0.137	-	-0.113
58	-	-0.171	-				

Supporting Information Table S3. Structure matrix extracted from the PCA. Coefficients ≥ ±0.1 were showed for all the proteins that resulted more indicative along the first three principal components.



Supporting Information Figure S1. 2-DE analysis of the *Lens culinaris* dry mature seed populations. Gel profiles of total proteins from: A) Capracotta 1 (reference map); B) Capracotta 2; C) Capracotta 3; D) Capracotta 4; E) Capracotta 5; F) Capracotta 6; G) Capracotta 7; H) Castelluccio di Norcia; I) Colfiorito; L) Rascino; M) Turca rossa; N) Canadese. Gels were stained with colloidal CBB R-250. An equal amount (400 mg) of total protein extracts was loaded in each gel.

Lentils biodiversity: the characterization of two local landraces

Vincenzo Viscosi, Manuela Ialicicco, Mariapina Rocco, Dalila Trupiano, Simona Arena, Donato Chiatante, Andrea Scaloni, Gabriella Stefania Scippa

Abstract — A multi-disciplinary approach was used to characterize two autochthonous lentil landraces from Molise region (Central Italy). Different mature seed populations for each landrace were provided by the Molise Germoplasm Bank at the University of Molise (Pesche, Italy), and analyzed at the morphological and molecular (DNA and protein) levels. Nuclear ISSR markers were used to assess genetic differences, whereas phenotypic variability was detected by biochemical (proteomics) and morphological analyses. The genetic and phenotypic diversity of the two lentil landraces were well assessed in relation to their geographical provenance, supporting further studies to identify landrace markers.

Index Terms — ISSR markers, *Lens culinaris*, seed morphology, proteomics.

1 Introduction

Lens culinaris Medik. has been cultivated around the Mediterranean basin since at least the seventh century B.C. and its cultivation area expanded to Middle East, Ethiopia and the Indian Subcontinent ([1], [2]). Local landraces are characterized by high genetic variability and high adaptation to different environmental conditions evolving in adaptive gene complexes [3]. However, in the industrialized countries the cultivation of many different local landraces has progressively decreased, becoming at high risk of genetic erosion ([4], [5]). In this paper, the diversity of two lentil landraces is analyzed to define and quantify differences between groups of populations coming from two geographical areas of Molise region (Central Italy). In particular, this study aims

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to deepen the knowledge about morphological, genetic and proteomic markers that differentiate local lentil landraces in relation to their provenance.

2 Material and Methods

Twelve lentil seed populations from Molise region (Central Italy) were studied in relation to their provenance: five from Conca Casale and seven from Capracotta. The total sample was analyzed by a multi-disciplinary approach, useful to characterize the phenotypical and genotypical traits of the two landraces. For the genetic analysis, nuclear ISSR markers were used as described in a previous paper [3], whereas the biochemical investigation was carried out on total seed proteins extracted according to the method of Rabilloud and resolved by 2DE [3]. For each population, gels were run in triplicate, and the mean value computed by software-assisted (PD-Quest) analysis was used to obtain a standardized matrix of the abundance (relative volume) of protein spots. One hundred seeds of each landrace population were used to measure eight morphological traits: area, perimeter, major axis length, minor axis length, and roundness, 100-seed weight, 100-seed volume and density (g/ml).

2.1 Statistical analysis

For molecular data, Principal Component Analysis was computed on Nei's genetic distance (1972) and the hierarchical partition of genetic variation among and within populations was obtained by means of the analysis of molecular variance (AMOVA). For biochemical and morphological data, a standardized matrix was subjected to univariate (ANOVA) and multivariate statistical analysis. Principal Component Analysis was computed on significant variables (detected by ANOVA) and the extracted Principal Components (eigenvalues > 1) were used in Canonical Variate Analysis.

3 Results

The results of ISSR analysis pointed out the genetic relationship between the two landraces. As shown in Fig. 1, the PCA highlighted a clear separation of the populations sampled in Conca Casale and Capracotta. In particular, along the first two PCs the total variance accounted for 45.57% and 18.04%, respectively. Molecular degree of differentiation between the two groups of populations (AMOVA) showed a significant molecular discrimination (PhiPT = 0.438; p = 0.001). Moreover, it resulted that the genetic variability was greater within (56%) than among (44%) groups of landrace populations.

The comparison of total seed proteomic maps of the Conca Casale and Capracotta populations revealed a total of 193 differentially expressed proteins. The biochemical data set (193 proteins) was subjected to ANOVA, to identify biochemical markers useful to distinguish lentil populations from different provenances. It resulted that 25 proteins were significant to discriminate Capracotta from Conca Casale lentils. PCA was computed on a correlation matrix, using these 25 significant proteins; the first two PCs explained 53.79%

and 11.62% of total variance, respectively, and the scatter plot of these two PCs indicated a clear distinction between the two groups of lentils (Fig. 2). Differences between the two landraces were tested by canonical variate analysis (CVA) computed on the extracted PCs. They were significantly discriminated (Wilks' $\lambda = 0.028$; df=5; $p < 0.0001$) as shown by the test of cross-validation (100% of cases were correctly classified).

The eight morphological variables were subjected to ANOVA in relation to population provenance. The two groups of lentil populations from Capracotta and Conca Casale were significantly discriminated by six morphological traits: seed density, roundness, volume, major axis length, perimeter and minor axis length. These six variables were used to compute a PCA: respectively, PC1 and PC2 explained 80.45% and 18.30% of total variance, highlighting a clear separation between landraces (Fig. 3). Then, the PCs were used in CVA and results indicated significant differences between the two population groups (Wilks' $\lambda = 0.047$; df = 2; sig.< 0.0001). Moreover, the test of cross-validations showed a high significance of the CVA reporting that 100% of cases was correctly classified.

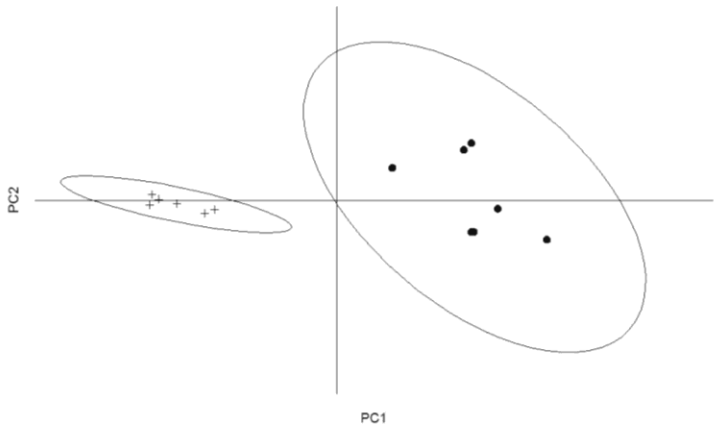

Fig. 1 – Scatter plot of specimens (cross = Conca Casale; point = Capracotta) ordered along the first two principal components; PCA from molecular data.

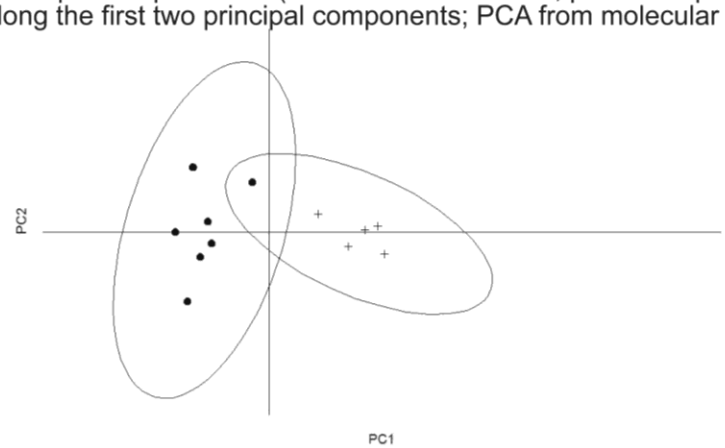

Fig. 2 – Scatter plot of specimens (cross = Conca Casale; point = Capracotta) ordered along the first two principal components; PCA computed on 25 proteins.

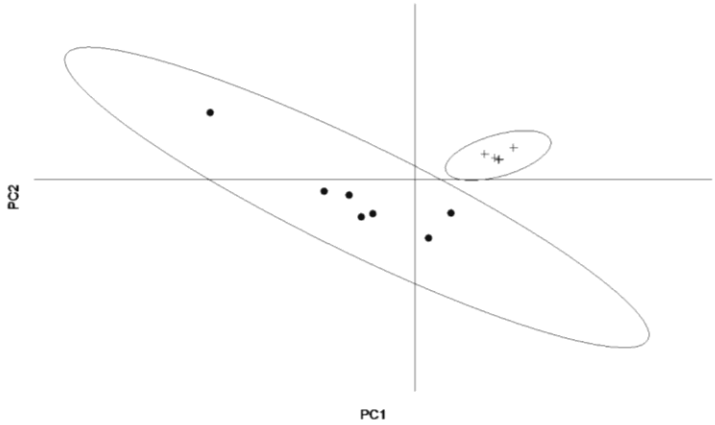

Fig. 3 – Scatter plot of specimens (cross = Conca Casale; point = Capracotta) ordered along the first two principal components; PCA computed on six morphological variables.

4 Conclusion

Autochthonous plant germplams, characterized by a wide genetic variability and high adaptation to different environmental conditions, are often more subjected to genetic erosion risks. In Italy, several different lentil landraces evolved thanks to the combination of different geographical characteristics.

The literature reports a wide variety of methods that have been used to investigate genetic similarities and relations among landraces of *L. culinaris* Medik.

Different methods have different powers of genetic resolution and provide different information: neutral DNA markers are useful tools to describe genetic relations in terms of time divergence [6], whereas phenotypic markers can provide information about adaptive responses to macro-environmental conditions [7].

In this study we used a combination of genetic and phenotypic analyses to characterize two autochthonous lentil landraces of two different provenances within a small region such as Molise.

The integration of genetic markers analysis with seed morphology and proteomic traits provided a high resolution approach to dissect lentil biodiversity [3]. The diversity between groups of populations, coming from two very close geographical areas, was well assessed and quantified. In addition, differences between the two local landraces were principally related to their sites of origin, where climate conditions and human activity may have selected the local accessions characterised by specific morphological and biochemical traits of seeds. Work is in progress to deepen the relation between these phenotypic markers and the environmental characteristics of the landrace provenance areas, and to identify the seed proteome markers.

References

[1] G. Ladizinsky, “The origin of lentil and its wild genepool”, *Euphytica*, vol. 28, pp. 179-187, 1979.

[2] Y. Duran, R. Fratini, P. Garcia and M. Perez de la Vega, “An intersubspecific genetic map of

Lens.", *Theor. Appl. Genet.*, vol. 108, pp. 1265-1273, 2004.
[3] G. S. Scippa, D. Trupiano, M. Rocco, V. Viscosi, M. Di Michele, A. D'Andrea and D. Chiatante, " An integrated approach to the characterization of two autochthonous lentil (*Lens culinaris*) landraces of Molise (south-central Italy)", *Heredity*, vol. 101, pp. 136-144, 2008.
[4] G. Ladizinsky, "Wild lentils". *Crit. Rev. Plant Sci.*, vol. 12, pp. 169-184, 1993.
[5] A. R. Piergiovanni, "The evolution of lentil (*Lens culinaris* Medik.) cultivation in Italy and its effects on the survival of autochthonous populations", *Genet. Resour. Crop Evol.*, vol. 47, pp. 305-314, 2000.
[6] H. Thiellement, N. Bahrman and C. Damerval, "Proteomics for genetic and physiological studies in plants", *Electrophoresis*, vol. 20, pp. 2013-2026, 1999.
[7] J. L. David, M. Zivy, M.L. Cardin and P. Brabant, "Protein evolution in dynamical managed population of wheat: adaptative responses to macro-environmental conditions",*Theor. Appl. Genet.*, vol. 95, pp. 932-941, 1997.

(Paper in preparation)

Seed proteome variation between two autochthon lentil (*Lens culinaris* Medik.) landraces

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Abstract

Unlike modern cultivars selected for their performance in specific environmental conditions, local landraces have a high genetic variability, which is widely recognized as an important genetic resource for plant breeding. To promote the survival of endangered lentil landraces, in previous studies we have investigated the genetic relationship between two ancient landrace cultivated in Molise region (Capracotta and Conca Casale, south-central Italy) and widely spread commercial varieties using an integrated approach consisting of studies at morphological, DNA and protein level.

The two local landraces were well differentiated from each other, and the Conca Casale landrace was separated from the commercial varieties at morphological, protein and DNA level. The Capracotta landrace, was well separated from the commercial varieties, except Castelluccio di Norcia, at DNA level showing a more complex and heterogeneous segregation at morphological and biochemical level. The correlation between morphological, DNA and protein data, illustrates that proteomics is a powerful tool with which to complement the analysis of biodiversity in ecotypes of a single plant species and to identify physiological and/or environmental markers. In the present work we used a proteomic approach to identify specific markers of the two autochthon lentil landraces, Capracotta and Conca Casale. The multivariate

statistical analyses carried out on 145 protein spots, resulting differentially expressed from proteome maps comparison, showed that 52 proteins were essential for population discrimination, thus determining their proposition as landrace markers.

Key words: Landrace markers / *Lens culinaris* / Plant germplasm/ Proteome map/ Seed / Storage proteins

1. Introduction

Lens culinaris Medik. has been cultivated around the Mediterranean Basin, since at least the seventh century B.C. Its cultivation area expanded to Middle East, Ethiopia and the Indian Subcontinent (Ladizinsky, 1979; Durán & Pérez de la Vega, 2004), raising in a myriad of different landraces (Erskine, 1997). Local landraces are characterized by high genetic variability, since their high adaptation to different environmental conditions evolved in adaptive gene complexes conserved by genetic linkage, natural or human selection.

However recently the cultivation of these landraces have progressively decreased in industrialized countries and a wide number local populations have disappeared while those still being cultivated are at a high risk of severe genetic erosion (Ladizinsky, 1993; Piergiovanni, 2000).

Characterization of *Lens culinaris* accessions has been applied at more levels, and several papers reported the values of phenotypic and genotypic traits as useful tools to distinguish among landraces and to investigate geographical patterns. In particular, morpho-agronomical characters, such as phenology or quantitative and qualitative data of flowers, leaves and seeds, were evaluated in relation to accession provenance and different climatic areas (Piergiovanni et al., 1998; Lázaro et al., 2001; Tullu et al., 2001; Poonam et al., 2006; Scippa et al., 2008; Sultana & Ghafoor, 2008; Toklu et al., 2009). Generally, these morphological parameters resulted useful in discriminating accessions and in describing differences and relationships. However it appeared that characters related to seed and productivity had most correlation with climate of the origin sites (Lázaro et al., 2001; Sultana & Ghafoor, 2008).

Geographical patterns in genetic variability of lentil landraces were assessed also by other markers at biochemical and DNA level (Durán & Pérez de la Vega, 2004). Isozymes (de la Rosa & Jouve, 1992; Rodríguez et al., 1999; Sultana & Ghafoor, 2008), seed storage proteins (de la Rosa & Jouve, 1992, Echeverrigaray et al., 1998; Piergiovanni & Taranto, 2005; Scippa et al., 2008; Zaccardelli et al., 2011), RAPDs (Sharma et al., 1995; Durán & Pérez de la Vega, 2004; Sultana & Ghafoor, 2008) and ISSRs (Alvarez et al., 1997; Sonnante & Pignone, 2001; Durán &

Pérez de la Vega, 2004; Scippa et al., 2008; Zaccardelli et al., 2011) are the most used markers for assessing lentil genetic variability.

Different methods have different powers of genetic resolution. In fact while 'neutral' DNA markers like microsatellites, describe relations between species in terms of time divergence ('molecular clock') (Thiellement et al., 1999), phenotypic markers like morpho-physiological traits can provide information about adaptive responses to macroenvironmental conditions (David et al., 1997). An innovative approach, such as proteomics, involving the comparison of several hundred to several thousand gene products resolved by two-dimensional gel electrophoresis of protein extracts, revealed to be particular interesting when used in investigating natural variations within plant species populations. Indeed, protein spots resolved by two-dimensional gel electrophoresis are de-facto genetic and physiological markers (Damerval et al., 1994; De Vienne et al., 1996) that can be used to assess genetic variability and to investigate adaptive responses to specific macro-environmental conditions.

In a previous study, we have assessed the genetic relationship between lentil landraces from Capracotta and Conca Casale, two little villages of Molise, and five commercial varieties using an integrated approach of genomic, morphological and proteomic analysis (Scippa et al., 2008). We found that the lentil populations of Conca Casale were well differentiated from the five commercial populations, at morphological, protein and DNA level, whereas all the Capracotta populations, clearly separated, at DNA level, from all the Conca Casale populations and from the commercial varieties, showing a more heterogeneous segregation at morphological and protein level.

Additionally we found a strong correlation between the genomic and proteomic data which strongly suggested that protein markers, resolved by two-dimensional electrophoresis, well characterize the different landraces, evidencing differences according to their physiological status. These findings were further supported by additional work where the lentil seed proteome was deciphered and the comparison between lentil populations from Capracotta and commercial varieties allowed the identification of specific landrace markers (Scippa et al., 2010).

The objective of the present study was to use a proteomic approach coupled with ANOVA statistical analysis to investigate (1) the genetic relationship between two local lentil landraces severely threatened by genetic erosion, and (2) to identify specific landrace protein markers.

2. Materials and methods

2.1 Plant materials

Lentil mature seeds of 13 lentil populations were provided by the Molise Germoplasm Bank at the University of Molise (Pesche), Italy; 7 of them (6 microsperma and 1 macrosperma) were

from Capracotta, and 6 were from Conca Casale, two small villages in the Molise region (south-central Italy). The two *L. culinaris* landraces used in this work have been previously characterized at genetic and morphological levels (Scippa et al., 2008). Three biological replicates consisting of almost 90 seeds (2.0 g) were analyzed for each lentil population.

2.2 Protein extraction

Total protein extraction was performed according to the method of Rabilloud (2000) with slight modifications, as previously described by Scippa et al. (2010). Briefly, for each lentil population, 2.0 g of mature seeds were ground in liquid nitrogen, and the fine powder was suspended in ice-cold acetone with 10% TCA, 0.07% β-mercaptoethanol and 1 mM PMSF. The mixture then was filtered through Miracloth, and proteins were precipitated at -20 °C for 4 hours. After incubation, the suspension was centrifuged at 35 000 g for 15 min. The protein pellet was washed twice with ice-cold acetone with 0.07% β-mercaptoethanol and 1 mM PMSF, dried under vacuum, and solubilized in rehydration sample buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 20 mM Tris, 1% (w/v) DTT, 0.2% (w/v) ampholine 3-10 and 0.15% (w/v) ampholine 5-7. Protein concentration was estimated according to Bradford assay (Bradford, 1976) using a SmartSpec Plus Spectophotometer (Bio-Rad) and bovine serum albumin as a standard.

2.3 2-DE

Protein pellets were washed with ice-cold methanol (once) and ice-cold acetone (three times), dried under reduced pressure and dissolved in IEF buffer (9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 20 mM DTT, 1% w/v carrier ampholytes pH 5-8; Bio-Rad, Hercules, CA, USA). IPG strips (18 cm pH 5-8, ReadyStrip, Bio-Rad) were rehydrated overnight with 460 µl of IEF buffer containing 600 µg of total proteins. Proteins were focused using a Protean IEF Cell (Bio-Rad) at 12 °C, applying the following voltages: 250 V (90min), 500 V (90min), 1000 V (180min) and 8000 V for a total of 55 kVh (Rocco et al., 2006). After focusing, the proteins were reduced by incubating the IPG strips with 1% w/v DTT for 15 min and alkylated with 2.5% w/v iodoacetamide in 10 mL of 50 mM Tris-HCl pH 8.8, 6 M urea, 30% w/v glycerol, 2% w/v SDS and a dash of bromophenol blue for 15 min. Electrophoresis in the second dimension was carried out on 12% polyacrylamide gels (18 x 24 cm x 1mm) with a Protean apparatus (Bio-Rad) in 25 mM Tris-HCl, pH 8.3, 1.92 M glycine and 1% w/v SDS, with 120 V applied for 12 h, until the dye front reached the bottom of the gel. 2-DE gels were stained with colloidal Coomassie G250 (Sigma). Samples were run in triplicate.

2.4 Image acquisition and statistical analysis

2-DE gels were scanned using a GS-800 calibrated densitometer (Bio-Rad). Image analysis was performed using the PDQuest software (Bio-Rad). Spot detection and matching between gels were performed automatically, followed by manual verification. Protein spots were annotated only if detectable in all gels. After normalization of the spot densities against the whole-gel densities, the percentage volume of each spot was averaged for three different replicates of each biological sample, and statistical Student's *t*-test analysis ($p<0.05$) was performed to find out significant changes in protein folding between samples. A two-fold change in normalized spot densities was considered indicative of a differential expression.

The mean volume of differential spots was used to obtain a standardized matrix of the abundance in order to compute univariate (ANOVA) and multivariate statistical analysis (Principal Component Analysis, Cluster Analysis, Canonical Variate Analysis).

2.5 In gel digestion and mass spectrometry

Protein spots of interest were manually excised from the gel and digested with trypsin (Talamo et al., 2003) for subsequent protein identification by MALDI-TOF-MS or nanoLC-ESI-LIT-MS/MS analysis. Digest aliquots were removed and subjected to a desalting/concentration step on µZipTipC18 (Millipore, Bedford, MA, USA) using ACN as eluent before MALDI-TOF-MS or nanoLC-ESI-LIT-MS/MS analysis.

In the first case, peptide mixtures were loaded on the MALDI target, using the dried droplet technique and a-cyano-4-hydroxycinnamic acid as matrix, and analyzed by using Voyager DE PRO and STR mass spectrometers (Applied Biosystems, Framingham, MA, USA), operating in positive ion reflectron mode, with an acceleration voltage of 20 kV, a nitrogen laser of 337 nm and a laser repetition rate of 4 Hz. The final mass spectra, measured over a mass range of 800-4000 Da and by averaging 50-300 laser shots, were elaborated using the DataExplorer 5.1 software (Applied Biosystems) and manually inspected to get the corresponding peak lists. Internal mass calibration was performed with peptides deriving from trypsin autoproteolysis.

In the second case, the digests were analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (Thermo) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon). Peptide mixtures were separated on an Easy C_{18} column (10 x 0.075 mm, 3 mm; Proxeon) using a linear gradient from 5 to 50% of ACN in 0.1% formic acid, over 60 min, at a flow rate of 300 nL/min. Spectra were acquired in the range of *m/z* 300-1800. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 3 min). The mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

2.6 Protein identification

MASCOT software package was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2008/05/03) in MALDI-TOF peptide mass fingerprinting experiments by using a mass tolerance value of 40-80 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. Candidates with a MASCOT score > 64, corresponding to $p<0.05$ for a significant identification, were further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

Sequest software package was used to identify spots unambiguously from an updated plant non-redundant sequence database (NCBI nr 2008/05/03) in ESI-LIT-MS/MS experiments by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. Candidates with more than two assigned peptides were ranked in ascending order according to consensus scores and false positive identifications minimized by filtration against four of the following criteria: Xcorr>2, ΔCn >0.2, Sp>400, rsp<5, ions>30% (Link et al., 1999). Where appropriate, protein identification was checked manually to provide for a false positive rate of <1% using Xcorr and ΔCn values described and validated elsewhere (Peng et al., 2003), and further evaluated by the comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

3. Results

Total protein extracts of mature seeds of 13 populations from two Molise lentil landraces (7 from Capracotta and 6 from Conca Casale) were separated by 2D electrophoresis. 2-DE gels were performed in a narrow linear range of pI 5-8 (Scippa et al., 2010) allowing a high resolution of the total proteins of *Lens culinaris* seed. The seed proteomic profile showed to be highly reproducible for all samples which were analysed in triplicate (Figure S1 in Supporting Information). Coomassie staining allowed to detect an average of 178 spots in the proteomic maps of Capracotta populations, whereas the mean number of detected spots for Conca Casale populations was 221. Once digitalized, the images of 2D gels were matched using population 1.2 as reference (Figure 1): the degree of similarity between reference and the other populations was within a 46%-85% range. To investigate quantitative differences in relative spot intensities, the proteomic maps of each lentil population were compared using a dedicated software. Statistical Student's *t*-test analysis ($p<0.05$) carried out with the PDQuest software detected 145 protein

spots whose expression difference was statistical meaningful.

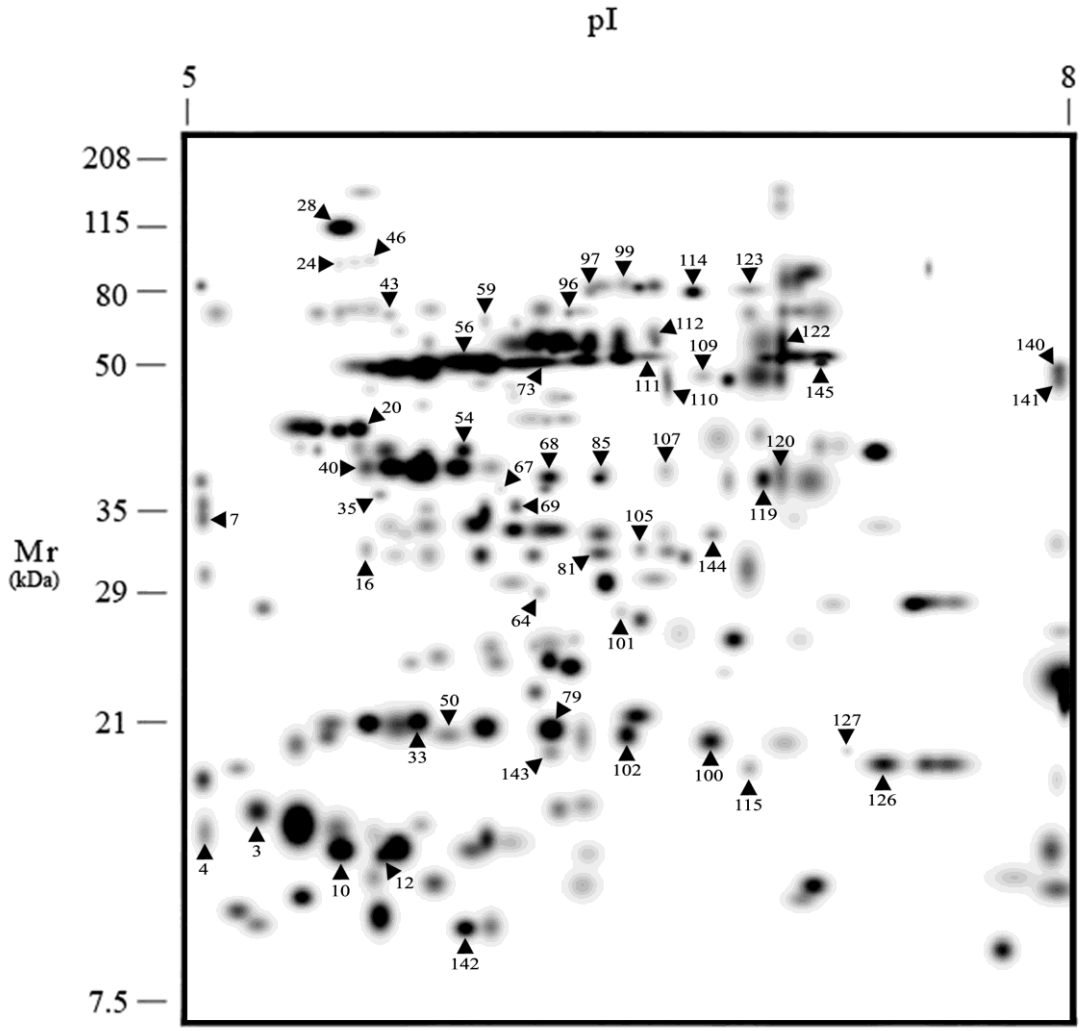

Figure 1. Two-dimensional electrophoresis reference map of total protein extract from *L. culinaris* mature seeds. 2-DE was performed on immobilized pH 5-8 strips, followed by the second-dimensional separation on 12% polyacrylamide gels. The 52 spots identified using mass spectrometry analysis are indicated with numbers in the map, corresponding to proteins listed in Table 1.

Quantitative data of 145 differentially expressed protein spots were subjected to ANOVA, to identify biochemical markers useful to distinguish between lentils from the two provenances. It resulted that 52 proteins were significant to discriminate Capracotta from Conca Casale populations (Supporting Information Table S1).

Principal Component Analysis that was computed on a var-covar matrix, using these 52 significant proteins: the first six principal components had eigenvalues ≥ 1.0 and explained the 94.47 of total variance. These were used to reduce the number of variables and to perform Canonical Variate Analysis on biochemical data. In fact, the differences between the two provenances of lentils, were tested and quantified by CVA: it resulted that the two populations

were significant discriminated (Wilks' $\lambda = 0.076$; df=6; p=0.002) and the test of cross-validation indicated high significance (100% of cases were correctly classified).

Subsequently, UPGMA (cluster analysis) was computed on correlation matrix for both landraces (Figure 2a) and proteins (Figure 2b). It resulted that the populations were clearly clustered by their provenance and the bootstrap (computed on 999 random permutations) indicated a significant separation between the populations from the two provenances. The proteins were grouped in two main clusters on the basis of geographical origin of lentils: cluster I grouped 44 spots (spots 3-145) that characterized Conca Casale populations for their higher quantity, including four spots (142, 143, 144, 145) which appeared to be specifically present only in gels of all specimens from Conca Casale (Figure S2 in Supporting Information); cluster II was constituted by 8 more expressed proteins (spots 4-123) in Capracotta; in particular the protein spots 4, 12, 119, 122 and 123 were completely absent in Conca Casale (Figure S2 in Supporting Information).

The fifty-two spots obtained by ANOVA were excised from gel and processed by digestion with trypsin in order to be analysed by MALDI-TOF PMF or nanoLC-ESI-LIT-MS/MS. On the whole, 86 proteins were identified as corresponding to 47 different protein accessions. In particular 31 spots were unequivocally identified, whereas 21 spots showed multiple identification (Table 1).

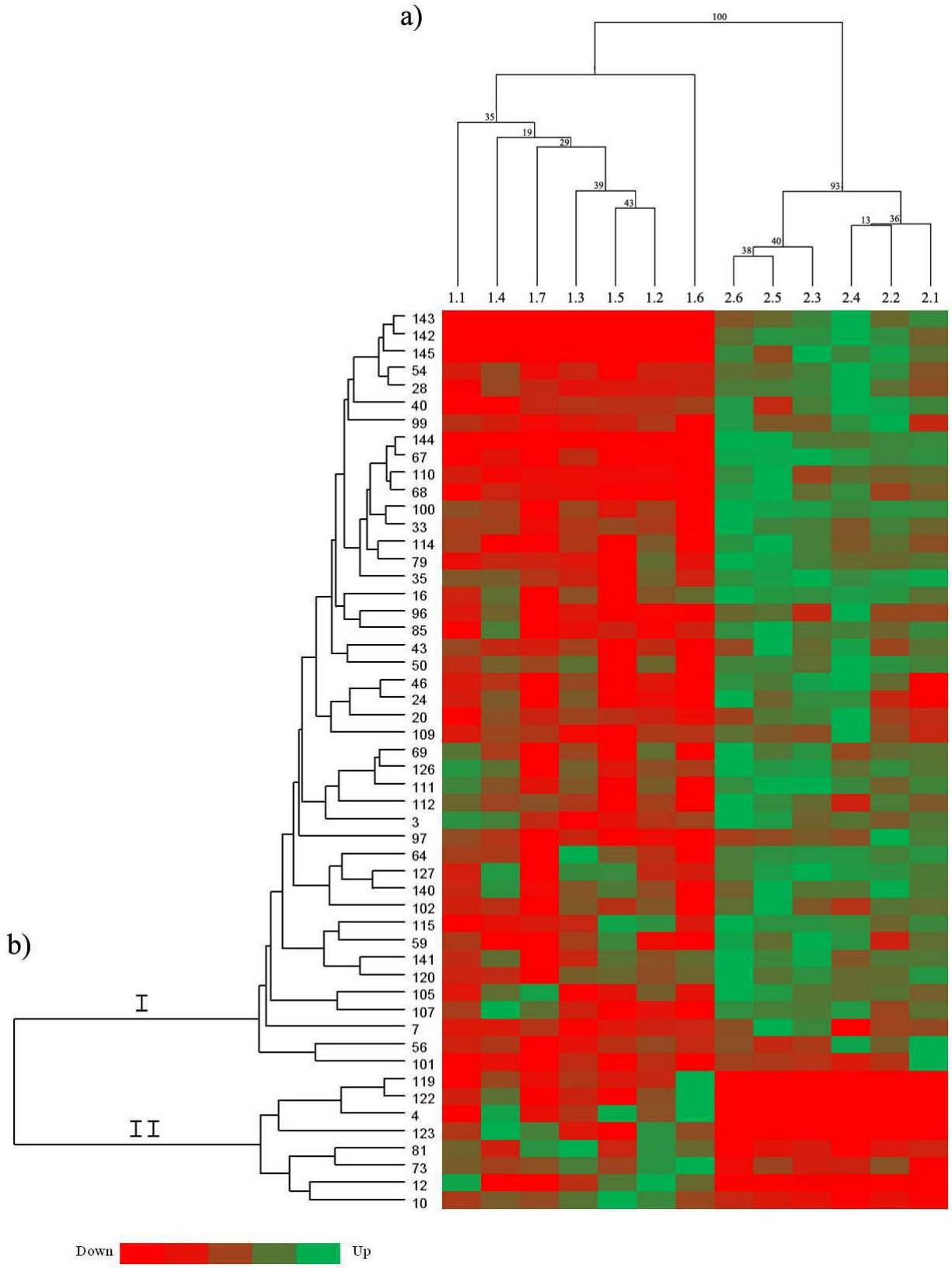

Figure 2. Cluster analysis computed on correlation matrix for the 13 different lentil populations (a) and the 52 differentially expressed proteins (ANOVA, $p \leq 0.05$) between Capracotta and Conca Casale lentil populations (b). Arabic numbers on the left side refer to proteins reported in Figure 1 and Table 1.

Table 1. List of proteins characterizing lentil landraces from Capracotta and Conca Casale. Spot number, accession number, protein name, experimental and theoretical Mw and pI values, method of identification, number of peptides matched, sequence coverage, Mascot score, organism and function are reported. Spot numbers refer to gel in Figure 1.

Spot number	Accession	Identification	MW (kDa)/pI_{th}	MW (kDa)/pI_{exp}	MS method	Matched peptides	Sequence Coverage	Mascot score	Species	Functional Class
3	gi29539111	Allergen Len c 1.0102	47.47 / 5.34	16.61 / 5.22	LCMS	2	6	16.08	*L. culinaris*	Protein destination and storage
	gi137581	Provicilin, type B	46.39 / 5.39		LCMS	2	3	9.26	*P. sativum*	Protein destination and storage
4	gi15236275	Cytochrome b6-f complex iron-sulfur subunit	22.53 / 8.58	15.96 / 5.06	LCMS	1	9	108.76	*A. thaliana*	Energy
7	gi1168189	14-3-3-like protein A	29.42 / 4.71	34.43 / 5.05	LCMS	2	12	147.50	*V. faba*	Others
	gi77997745	Seed maturation protein	26.49 / 4.82		LCMS	3	14	132.93	*M. truncatula*	Disease/defence
10	gi75243366	Allergen Len c 1.0101	47.83 / 5.35	15.35 / 5.50	PMF	9	18	105	*L. culinaris*	Protein destination and storage
12	gi137582	Vicilin	52.23 / 5.39	15.10 / 5.60	LCMS	13	20	379.02	*P. sativum*	Protein destination and storage
16	gi14488168	Lectin	27.89 / 5.34	32.19 / 5.57	PMF	5	22	77	*L. culinaris*	Disease/defence
20	gi126170	Legumin J	56.90 / 5.70	42.90 / 5.54	LCMS	4	17	23.88	*P. sativum*	Protein destination and storage
	gi542002	Legumin, type B	32.73 / 5.23		LCMS	2	10	11.41	*V. faba*	Protein destination and storage
	gi282925	Legumin K	56.28 / 5.65		LCMS	5	18	32.48	*P. sativum*	Protein destination and storage
24	gi15292925	Heat shock cognate 70 kDa protein 3	71.15 / 4.96	91.85 / 5.48	LCMS	5	8	160	*A. thaliana*	Disease/defence
	gi123620	Heat shock cognate 70 kDa protein 2	70.71 /5.07		LCMS	16	23	522	*S. lycopersicum*	Disease/defence
28	gi30690736	Cupin family protein	71.44 / 9.46	115.00 / 5.50	LCMS	18	25	692.62	*A. thaliana*	Protein destination and storage
33	gi29539111	Allergen Len c 1.0102	47.47 / 5.34	21.00 / 5.80	PMF	17	31	186	*L. culinaris*	Protein destination and storage
35	gi22008	Legumin A2 primary translation product	56.68 / 6.15	36.64 / 5.60	LCMS	5	12	178.71	*V. faba*	Protein destination and storage
	gi255552295	Short chain dehydrogenase	31.66 / 6.39		LCMS	6	26	368.43	*R. communis*	Others
40	gi126168	Legumin A	57.62 / 6.11	39.23 / 5.56	LCMS	3	4	15.49	*P. sativum*	Protein destination and storage
	gi126161	Legumin A2	59.27 / 6.21		LCMS	5	7	25.91	*P. sativum*	Protein destination and storage
43	gi2116558	F1 ATPase	60.15 / 6.63	67.64 / 5.66	PMF	13	23	129	*P. sativum*	Energy
46	gi15292925	Heat shock cognate 70 kDa protein 3	71.15 / 4.96	93.48 / 5.58	LCMS	2	4	91	*A. thaliana*	Disease/defence
	gi123620	Heat shock cognate 70 kDa protein 2	70.71 /5.07		LCMS	2	4	98	*S. lycopersicum*	Disease/defence
50	gi29539111	Allergen Len c 1.0102	47.47 / 5.34	20.08 / 5.88	LCMS	5	10	38.42	*L. culinaris*	Protein destination and storage
	gi29539109	Allergen Len c 1.0101	47.83 / 5.35		LCMS	5	9	37.73	*L. culinaris*	Protein destination and storage
	gi87162566	Cupin, RmlC-type	53.15 / 5.42		LCMS	4	7	34.01	*M. truncatula*	Protein destination and storage
	gi758248	Vicilin precursor	49.52 / 5.33		LCMS	4	7	32.72	*P. sativum*	Protein destination and storage
54	gi758248	Vicilin precursor	49.52 / 5.33	40.82 / 5.93	LCMS	2	6	21.58	*P. sativum*	Protein destination and storage
	gi137579	Provicilin, type A	31.54 / 5.57		LCMS	2	16	17.64	*P. sativum*	Protein destination and storage
	gi87162566	Cupin, RmlC-type	53.15 / 5.42		LCMS	2	5	11.94	*M. truncatula*	Protein destination and storage
	gi259474	Legumin propolypeptide alpha chain	31.96 / 5.11		LCMS	3	21	19.00	*Papilionoideae*	Protein destination and storage
	gi126171	Legumin K alpha and beta chain	39.78 / 5.38		LCMS	2	3	10.55	*P. sativum*	Protein destination and storage

Spot number	Accession	Identification	MW (kDa)/pI_{th}	MW (kDa)/pI_{exp}	MS method	Matched peptides	Sequence Coverage	Mascot score	Species	Functional Class
56	gi29539111	Allergen Len c 1.0102	47.47 / 5.34	50.48 / 5.93	LCMS	7	16	54.26	*L. culinaris*	Protein destination and storage
	gi29539109	Allergen Len c 1.0101	47.83 / 5.35		LCMS	7	16	53.04	*L. culinaris*	Protein destination and storage
	gi137582	Vicilin	52.23 / 5.39		LCMS	8	18	61.43	*P. sativum*	Protein destination and storage
	gi137584	Vicilin	52.69 / 5.77		LCMS	4	7	20.94	*V. faba*	Protein destination and storage
	gi137581	Provicilin, type B	46.39 / 5.39		LCMS	4	9	21.03	*P. sativum*	Protein destination and storage
	gi82173888	Provicilin	51.42 / 6.04		LCMS	5	11	24.04	*C. arietinum*	Protein destination and storage
	gi2765097	P54 protein	54.66 / 6.05		LCMS	6	15	51.30	*P. sativum*	Disease/defence
59	gi42521309	Enolase	47.72 / 5.31	65.01 / 5.99	LCMS	5	17	269.44	*G. max*	Energy
	gi255583291	Xylose isomerase	45.67 / 5.48		LCMS	3	9	130.67	*R. communis*	Metabolism
	gi137582	Vicilin	52.23 / 5.39		LCMS	5	14	192.31	*P. sativum*	Protein destination and storage
64	gi259470	Ferritin	23.58 / 5.56	29.04 / 6.15	LCMS	8	47	283.16	*P. sativum*	Protein destination and storage
67	gi137582	Vicilin	52.23 / 5.39	37.21 / 6.04	LCMS	8	18	262.09	*P. sativum*	Protein destination and storage
68	gi75243366	Allergen Len c 1.0101	47.83 / 5.35	38.33 / 6.19	PMF	18	41	225	*L. culinaris*	Protein destination and storage
69	gi137582	Vicilin	52.23 / 5.39	35.61 / 6.08	LCMS	12	31	514.05	*P. sativum*	Protein destination and storage
73	gi170010	Late embryongenesis abundant protein	50.64 / 6.33	50.38 / 6.16	LCMS	14	31	638.37	*G. max*	Disease/defence
	gi137582	Vicilin	52.23 / 5.39		LCMS	10	23	311.51	*P. sativum*	Protein destination and storage
79	gi7339555	Vicilin 47k precursor	17.82 / 9.24	20.51 / 6.19	LCMS	4	22	28.78	*P. sativum*	Protein destination and storage
81	gi137582	Vicilin	52.23 / 5.39	31.96 / 6.34	LCMS	3	7	16.70	*P. sativum*	Protein destination and storage
85	gi29539109	Allergen Len c 1.0101	47.83 / 5.35	38.48 / 6.34	PMF	11	27	101	*L. culinaris*	Protein destination and storage
96	gi75184075	Convicilin	60.13 / 5.56	68.82 / 6.24	PMF	12	30	137	*L. culinaris*	Protein destination and storage
97	gi7688242	Convicilin	60.13 / 5.56	77.72 / 6.31	LCMS	7	17	335.13	*L. culinaris*	Protein destination and storage
	gi187710977	Group 3 LEA protein	47.16 / 5.37		LCMS	3	9	271.25	*M. sativa*	Disease/defence
	gi137582	Vicilin	52.23 / 5.39		LCMS	8	19	228.24	*P. sativum*	Protein destination and storage
99	gi126170	Legumin J	56.90 / 5.70	81.56 / 6.41	LCMS	8	24	239.91	*P. sativum*	Protein destination and storage
	gi137582	Vicilin	52.23 / 5.39		LCMS	7	18	207.75	*P. sativum*	Protein destination and storage
100	gi29539109	Allergen Len c 1.0101	47.83 / 5.35	19.73 / 6.68	PMF	11	25	110	*L. culinaris*	Protein destination and storage
101	gi2765097	P54 protein	54.66 / 6.05	27.71 / 6.40	PMF	18	36	143	*P. sativum*	Disease/defence
	gi33285914	Putative dehydroascorbate reductase	12.04 / 6.15		LCMS	2	15	15.30	*B. rapa*	Disease/defence
102	gi122189405	Cyclin-like F-box	45.38 / 9.10	20.20 / 6.42	PMF	8	25	69	*M. truncatula*	Cell growth/division
105	gi195658029	Lipoprotein	27.05 / 6.42	32.21 / 6.47	LCMS	10	62	262.33	*Z. mais*	Others
107	gi30687999	Ribulose bisphosphate carboxylase/oxygenase activase	48.50 / 7.55	38.82 / 6.54	LCMS	5	21	249.06	*A. thaliana*	Energy
	gi137582	Vicilin	52.23 / 5.39		LCMS	7	14	276.12	*P. sativum*	Protein destination and storage
109	gi11992263	Glycinin subunit G7	60.49 / 6.49	48.85 / 6.66	LCMS	8	10	175.22	*G. max*	Protein destination and storage
110	gi758248	Vicilin precursor	49.52 / 5.33	47.54 / 6.55	LCMS	5	13	30.07	*P. sativum*	Protein destination and storage
111	gi137582	Vicilin	52.23 / 5.39	52.62 / 6.49	LCMS	10	33	364.32	*P. sativum*	Protein destination and storage
112	gi7688242	Convicilin	60.13 / 5.56	60.13 / 6.51	LCMS	4	11	213.58	*L. culinaris*	Protein destination and storage
	gi137582	Vicilin	52.23 / 5.39		LCMS	11	26	462.99	*P. sativum*	Protein destination and storage
114	gi187710977	Group 3 LEA protein	47.16 / 5.37	78.06 / 6.63	LCMS	21	45	639.83	*M. sativa*	Disease/defence

Spot number	Accession	Identification	MW (kDa)/pI_{th}	MW (kDa)/pI_{exp}	MS method	Matched peptides	Sequence Coverage	Mascot score	Species	Functional Class
115	gi110611256	Lectin	29.52 / 5.10	18.24 / 6.80	LCMS	2	8	10.86	*C. arietinum*	Disease/defence
119	gi29539109	Allergen Len c 1.0101	47.83 / 5.35	38.10 / 6.84	PMF	9	22	93	*L. culinaris*	Protein destination and storage
120	gi255552295	Short chain dehydrogenase	31.66 / 6.39	38.50 / 6.90	LCMS	26	75	1185.87	*R. communis*	Others
	gi137582	Vicilin	52.23 / 5.39		LCMS	8	19	307.17	*P. sativum*	Protein destination and storage
122	gi137582	Vicilin	52.23 / 5.39	57.66 / 6.91	LCMS	6	16	228.26	*P. sativum*	Protein destination and storage
123	gi187710977	Group 3 LEA protein	47.16 / 5.37	79.09 / 6.80	LCMS	4	11	201.50	*M. sativa*	Disease/defence
126	gi29539109	Allergen Len c 1.0101	47.83 / 5.35	18.40 / 7.27	PMF	10	20	98	*L. culinaris*	Protein destination and storage
127	gi29539109	Allergen Len c 1.0101	47.83 / 5.35	19.17 / 7.16	PMF	10	24	73	*L. culinaris*	Protein destination and storage
140	gi87240526	Peptidase A1, pepsin	48.15 / 8.87	49.72 / 7.83	LCMS	8	23	449.91	*M. truncatula*	Protein destination and storage
141	gi1431629	Pectinacetylesterase precursor	43.82 / 8.83	48.31 / 7.83	LCMS	3	13	179.64	*V. radiata*	Cell structure
	gi87240526	Peptidase A1, pepsin	48.15 / 8.87		LCMS	7	20	325.70	*M. truncatula*	Protein destination and storage
142	gi254938914	Bowman-Birk inhibitor	12.78 / 6.77	9.71 / 5.93	LCMS	2	14	100.23	*L. culinaris*	Disease/defence
143	gi137582	Vicilin	49.34 /5.33	19.05 / 6.19	LCMS	2	5	58	*P. sativum*	Protein destination and storage
	gi123555	18.1 kDa class I heat shock protein	18.09 /5.83		LCMS	5	30	156	*P. sativum*	Disease/defence
144	gi137582	Vicilin	52.23 / 5.39	33.46 / 6.69	LCMS	4	9	187.91	*P. sativum*	Protein destination and storage
145	gi137582	Vicilin	52.23 / 5.39	51.60 / 7.07	LCMS	9	24	225.29	*P. sativum*	Protein destination and storage
	gi11363146	IAA-protein conjugate	35.52 / 6.20		LCMS	2	4	125.01	*P. vulgaris*	Others

Identified proteins were categorized according to their functions in seven different categories using the Bevan's classification (1998). As shown in Figure 3, the most highly represented functional class was protein destination and storage (67%), following by proteins associated with disease/defense (19%), energy (5%), metabolism (1%), cell structure (1%), cell growth/division (1%), and others (6%).

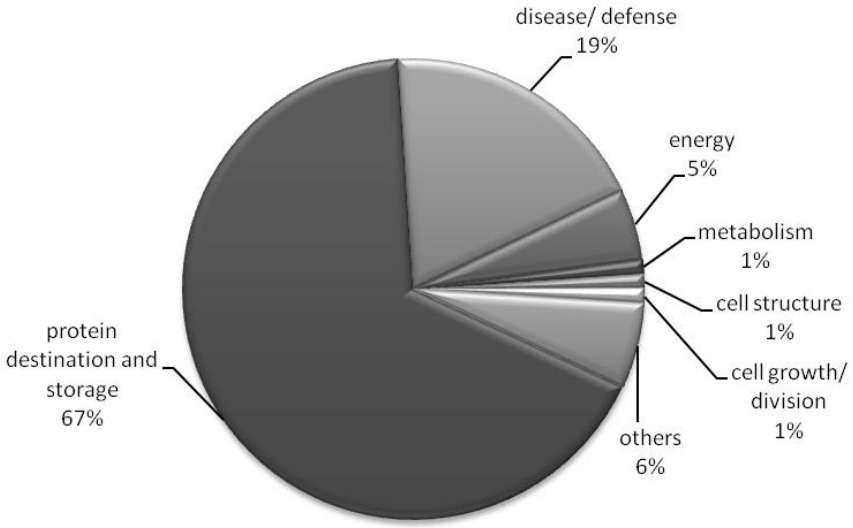

Figure 3. Classification of the proteins identified according to their biological function.

4. Discussion

Due to its high seed protein content, lentil is considered to be one of the most nutrient grain legumes, constituting an important protein source for human diet in several rural communities (Zohary, 1995).

In Italy, lentil cultivation is essentially based on local varieties whose farming areas include solely some marginal lands of south-central Apennines and small islands (Piergiovanni, 2000). These lentil landraces were selected both by farmers and by the agro-environmental conditions in which they have been grown over the centuries, developing a high adaptation to different local environments (Torricelli et al., 2012). A wide variety of methods has been used to investigate genetic similarities and relations among *L. culinaris* Medik. landraces. Morphological and phenological studies have revealed significant differences in Italian lentil populations (Gallo et al., 1997), however still few studies have evaluated genetic variation at molecular (Sonnante & Pignone, 2001; Scippa et al., 2008) or biochemical level (Senatore et al., 1992; Scippa et al., 2010).

The genetic variability within and among different lentil populations have been assessed by using seed storage proteins (de la Rosa & Jouve, 1992; Echeverrigaray et al., 1998), and

proteomic analysis (Scippa et al., 2008, 2010). Unlike one-dimensional electrophoresis, which separates proteins according to their molecular weight, two-dimensional electrophoresis resolves proteins based on isoelectric point and molecular weight, allowing the examination of a broad spectrum of proteins and, consequently, a substantially larger number of protein-encoding loci. Moreover, two dimensional electrophoresis is a high-resolution technique that separates thousands of genetic products (protein spots) on a single gel and detects isomorphs, polymorphisms and changes such as post-translational modifications (that is phosphorylation, glycosylation, acetylation and methylation) induced for instance, by precise ecological situations experienced by individuals (David et al., 1997; Chevalier et al., 2004). Furthermore, two-dimensional electrophoresis coupled with mass spectrometry represents a powerful tool with which to identify 'physiological markers', and to determine if differences between populations are due to adaptation to particular environments (David et al., 1997).

In a previous study, we have investigated the genetic relationship between the two ancient landrace cultivated in Capracotta and Conca Casale (Molise, south-central Italy) and several widely distributed commercial varieties and found that these two landraces are clearly differentiated at morphological, genetic and biochemical level (Scippa et al., 2008; Viscosi et al., 2010).

In the present study, we have used proteomics to further investigate the variation of protein patterns in mature seed of the two autochthonous lentil landraces from Molise (Capracotta and Conca Casale) with aim to identify specific protein markers.

Thirteen populations, seven from Capracotta and six from Conca Casale, were chosen to be studied by 2-DE and a comparison of their proteome maps revealed 145 protein spots as differentially expressed between populations. Out of these 145 variable expressed spots, 52 resulted significant for populations discrimination by multivariate statistical analysis (ANOVA).

Eight spots distinguished all Capracotta lentil populations for their high expression whereas remaining spots, in a number of 44, were qualitatively and/or quantitatively more abundant in Conca Casale populations.

The majority of spots discriminating between the two landraces were associated with storage proteins, namely 7S (vicilins and convicilins) and 11S (legumins) globulins which represent the most important storage proteins in legume seeds (Croy et al., 1980). However, while Capracotta differentiated for the high level of six spots associated with 7S globulins, Conca Casale distinguished for the significant abundance of twenty five 7S and six 11S globulins.

Seed storage proteins have the pivotal role of providing the seedling a source of amino acids and nitrogen for metabolism during seed germination and seedling growth (Lambert & Yarwood, 1992; Spencer, 1984; Shewry et al., 1995). However, they have been also associated with the role

in defending seeds against bruchids and pathogens (Sales et al., 2000). In a previous work we suggested that storage proteins identified in lentil seed proteome are mainly represented by 7S and 11S globulins which are characterised by a high degree of heterogeneity (Scippa et al., 2010). Additionally, we showed that similarly to pea and contrarily to other legumes (Bourgeois et al., 2009), the number of spots identified as 7S proteins was markedly higher compared with the number of the 11S globulins, and that Capracotta landrace distinguished from the other commercial varieties for the poor legumin levels (Scippa et al., 2010). Data reported in the present work confirm these previous findings highlighting that the high degree of protein storage heterogeneity is an important factor in landraces discrimination. Furthermore, here we show that the two local landraces are significantly differentiated by different level of 7S and 11S globulin. In fact, although Capracotta landrace was previously well distinguished from commercial varieties for the abundance of 7S globulins (Scippa et al., 2010), in the present work it appears characterised by a meaningful lower level of protein storage heterogeneity compared to Conca Casale. Indeed Conca Casale was well distinguished from Capracotta for the abundance of twenty five 7S and six 11S globulin. Moreover, because the 7S/11S ratio is an important determinant of the seed nutritional quality (Bourgeois et al., 2009), the particular nature of lentil seed landraces protein composition had to be precisely analyzed and may provide specific end-uses for its proteins.

However, Capracotta is also distinguished from Conca Casale populations for the higher level of two spots (n. 73 and 123) identified as LEA (Late Embryogenesis Abundant) proteins, and a spot (n. 4) corresponding to a cytochrome b6-f complex iron-sulfur subunit. LEA accumulation in plant embryos during the latter stages of seed maturation is correlated with the acquisition of desiccation tolerance (Bartels et al., 1988; Blackman et al., 1995; Cuming, 1999; Buitink et al., 2002).

The cytochrome b6-f complex iron-sulfur subunit, a central component of the respiratory and photosynthetic electron transport chains, has been associated with multiple roles beyond energy transduction, such as regulating gene expression (de Vitry et al., 2004) and stress tolerance, including drought. In fact it has been found to be induced by a combination of drought and heat stress in maize (Hu et al., 2010) and be overexpressed in a more drought tolerant genotype of groundnut (Katam et al., 2007).

Other proteins related to the stress defence, seed maturation and energy production, differentiated Conca Casale for their qualitative and quantitative high expression from Capracotta populations, and may be proposed as important landrace markers.

Two group 3 LEA proteins (spot n. 97 and 114), three heat shock proteins (spots n. 24, 46 and 143), a putative dehydroascorbate reductase (spot n. 101), three cupins (spot n. 28, 50 and 54),

two P54 proteins (spot n. 56 and 101) may ensure acquisition of Conca Casale lentil seeds desiccation tolerance. The latter proteins seem to exert a protective function of the chromatin structure against desiccation (Chiatante et al., 1995; Castillo et al., 2000, 2005), whereas cupins, classified as storage proteins, beside in stress defence can be involved in such other relevant functions as seed germination (Dunwell et al., 2000; Dunwell et al., 2004). In this context, also ferritin (spot n. 64) was recently demonstrated to be involved in protecting seeds against oxidative damage, and in facilitating seed germination (Ravet et al., 2009).

Other proteins seem to guarantee Conca Casale lentil seed response to biotic stresses. One spot (spot n. 7) corresponded to 14-3-3 like protein A, while two spots (n. 16 and 115) were identified as lectin accessions. 14-3-3 proteins are known to function in protein-protein interactions that mediate signal transduction pathways regulating many biological processes, such as metabolism, hormone and light signalling, transcription, cell-cycle control, protein trafficking, and stress responses (Sehnke et al., 2002; Roberts, 2003; Morrison, 2009; Gökirmak et al.,2010; Oh, 2010). Owing to their binding to bacterial polysaccharides, lectins have been associated with plant pathogens response, but are also involved in seed germination/conservation (Sharon & Lis, 1990; Roopashree et al., 2006).

Similarly, the Bowman-Birk protease inhibitor (spot n. 142) may ensure a defense mechanism of the seed against insect midgut proteases (de Azevedo Pereira et al., 2007). Bowman-Birk serine protease inhibitors are a family of small plant proteins, whose physiological role has not been ascertained as yet, while chemopreventive anticarcinogenic properties have repeatedly been claimed. Like many other cotyledonary proteins, BBIs are the products of a multigene family within the same species (Mello et al., 2003; Deshimaru et al., 2004) and consequently several isoforms have been identified (Prakash et al., 1996; Sonnante et al., 2005). There have been various hypotheses on the physiological function of BBIs, including defence and protection, developmentally regulatory and sulphur-storage roles, with no conclusive definition as yet (Mosolov & Valueva, 2005). Lentil seeds contain various BBI isoforms which are the products of few genes (Sonnante et al., 2005). In our previous paper we showed that Capracotta landrace was distinguished from the commercial varieties for the abundance of a BBI (Scippa et al., 2010), whereas here we find that Conca Casale is characterised by a BBI that is absent in Capracotta. Based on these data it can be suggested that the two local landraces express different BBI isoforms which are probably lacking in the commercial varieties.

Findings on the involvement of this molecule in the prevention of tumorigenesis suggest a possible positive contribution of protease inhibitors to the nutritional value of legume seeds (Kennedy, 1998; Qi et al., 2005).

Proteins that become abundant in seed embryos and may control embryogenesis, such as seed

maturation protein (spot n. 7) and cyclin-like F-box (spot n. 102), were also identified (Lechner et al., 2006) to be more expressed in Conca Casale seed landrace. Furthermore, a high expression of proteins involved in energy metabolism and nutrients production was associated to Conca Casale seed landrace. In fact an enolase (spot n. 59), a F1 ATPase (spot n. 43), two peptidases A1 (spots n. 140 and 141) implicated in proteolysis of storage proteins during seed germination (Spencer & Spencer, 1974), and a lipoprotein (spot n. 105) associated to the formation of cell membranes and the transport of fatty acids or their CoA derivatives, (Douliez et al., 2000) were more abundant in this landrace compared to Capracotta. Overall the higher expression of factors involved in energy and metabolism seems to suggest that Conca Casale seeds are better-equipped in quickly releasing stored energy and nutrients (storage proteins) for rapid growth of plant tissues, as during the early germination steps, and contain stored enzymes to allow a rapid resumption of essential metabolic activities.

This hypothesis may be confirmed by the qualitative expression of a spot (n.145) identified as IAA-protein conjugate, the most abundant protein covalently modified by the phytohormone indoleacetic acid (Walz et al., 2002). Indoleacetic acid-modified proteins represent a distinct class of conjugated phytohormones and appear to be the major auxin storage forms in seeds (Walz et al., 2002). Expression and modification of IAA-protein conjugate is correlated to the developmental period of rapid growth during seed development, whereas its degradation occurs during germination to free active IAA (Ljung et al., 2001).

In conclusion, in this work the lentil seed proteome composition has been compared between two autochthon ecotypes with the identification of important protein markers . The two ecotypes were significantly differentiated by a high heterogeneity of storage proteins; however a number of other proteins potentially involved in stress tolerance, seed conservation and germination was also observed. Our results suggest that Conca Casale lentil ecotype compared to Capracotta is better equipped (i) to face various abiotic/biotic stresses, (ii) to provide various nutritional protein and non-protein compounds for the growing plantlet, and (iii) to enhance germination by ready-protein machineries present in the seed. This study provides additional support on the use of the proteomic approaches to assess genetic distances and phylogenetic relationships between various plant ecotypes.

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References

Alvarez, M.T., Garcia, P., Pérez de la Vega, M. (1997) RAPD polymorphism in Spanish lentil landraces and cultivars. *Journal of Genetics* and *Breeding*, **51**, 91-96.

Bartels, D., Singh, M., Salamini, F. (1988) Onset of desiccation tolerance during development of the barley embryo. *Planta*, **175**, 485-492.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., Drost, L., Ridley, P., Hudson, S.A., Patel, K., Murphy, G., Piffanelli, P., Wedler, H., Wedler, E., Wambutt, R., Weitzenegger, T., Pohl, T.M., Terryn, N., Gielen, J., Villarroel, R., De Clerck, R., Van Montagu, M., Lecharny, A., Auborg, S.,Gy, I., Kreis, M., Lao, N., Kavanagh, T., Hempel, S., Kotter, P., Entian, K.D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Silvey, M., James, R., Montfort, A., Pons, A., Puigdomenech, P., Douka, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, B., Hilbert, H., Düsterhöft, A., Moores, T., Jones, J.D., Eneva, T., Palme, K., Benes, V., Rechman, S., Ansorge, W., Cooke, R., Berger, C., Delseny, M., Voet, M., Volckaert, G., Mewes, H.W., Klosterman, S., Schueller, C., Chalwatzis, N. (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature*, **391**, 485-488.

Blackman, S.A., Obendorf, R.L., Leopold, A.C. (1995) Desiccation tolerance in developing soybean seeds-the role of stress proteins. *Physiologia Plantarum*, **93**, 630-638.

Bourgeois, M., Jacquin, F., Savois, V., Sommerer, N., Labas, V., Henry, C., Burstin, J. (2009) Dissecting the proteome of pea mature seeds reveals the phenotypic plasticity of seed protein composition. *Proteomics*, **9**, 254-271.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. *Analytical Biochemistry*, **72**, 248-254.

Buitink, J., Hoekstra, F.A., Leprince, O. (2002) Biochemistry and biophysics of tolerance systems. In: *Desiccation and Survival in Plants: Drying Without Dying* (ed. M. Black & H.W. Pritchard) CABI Publishing, Oxon, UK, 293-318.

Castillo, J., Rodrigo, M.I., Márquez, J.A., Zúñiga, A., Franco, L. (2000) A pea nuclear protein that is induced by dehydration belongs to the vicilin superfamily. *European Journal of*

Biochemistry, **267**, 2156-2165.

Castillo, J., Genoves, A., Franco, L., Rodrigo, M.I., (2005) A multifunctional bicupin serves as precursor for a chromosomal protein of *Pisum sativum* L. seeds. *Journal of Experimental Botany*, **56**, 3159-3169.

Chevalier, F., Martin, O., Rofidal, V., Devauchelle, A.D., Barteau, S., Sommerer, N., Rossignol, M. (2004) Proteomic investigation of natural variation between *Arabidopsis* ecotypes. *Proteomics*, **4**, 1372-1381.

Chiatante, D., Onelli, E., Patrignani, G., Scippa, G.S. (1995) Localization of a nuclear protein (QP47) in embryonic meristems during seed maturation and germination and its distribution among crop plants. *Journal of Experimental Botany*, **46**, 815-821.

Croy, R.R.D., Gatehouse, J.A., I., Evans, I.M., Boulter, D. (1980) Characterisation of the storage protein subunits synthesised in vitro by polyribosomes and RNA from developing pea (*Pisum sativum* L.). *Planta*, **148**, 49-56.

Cuming, A.C. (1999) LEA proteins. In: *Seed Proteins* (ed. P.R. Shewry & R. Casey) Kluwer Academic Press, Dordrecht, The Netherlands, 753-780.

Damerval, C., Maurice, A., Josse, JM., De Vienne, D. (1994) Quantitative trait loci underlying gene product variation: a novel perspective for analyzing regulation of genome expression. *Genetics*, **137**, 289-301.

David, J.L., Zivy, M., Cardin, M.L., Brabant, P. (1997) Protein evolution in dynamical managed population of wheat: adaptative responses to macro-environmental conditions. *Theoretical and Applied Genetics*, **95**, 932-941.

de Azevedo Pereira, R., Valencia-Jiménez, A., Magalhães, C.P., Prates, M.V., Melo, J.A., de Lima, L.M., de Sales, M.P., Tempel Nakasu, E.Y., da Silva, M.C., Grossi-de-Sá, M.F. (2007) Effect of a Bowman-Birk proteinase inhibitor from *Phaseolus coccineus* on *Hypothenemus hampei* gut proteinases in vitro. *Journal of Agricultural and Food Chemistry*, **55**, 10714-10719.

de la Rosa, L. & Jouve, N. (1992) Genetic variation for isozyme genes and proteins in Spanish

primitive cultivars and wild subspecies of genus *Lens. Euphytica*, **59**, 181-187.

De Vienne, D., Burstin, J., Gerber, S., Leonardi, A., Le Guilloux, M., Murigneux. A., Beckert, M., Bahrman, N., Damerval, C., Zivy, M. (1996) Two-dimensional electrophoresis of proteins as a source of monogenic and codominant markers for population genetics and mapping the expressedgenome. *Heredity*, **76**, 166-177.

de Vitry**,** C**.,** Ouyang**,** Y**.,** Finazzi**,** G**.,** Wollman**,** F**.**A**.,** Kallas**,** T. (2004) The chloroplast Rieske iron-sulfur protein. At the crossroad of electron transport and signal transduction. *The Journal of Biological Chemistry*, **279,** 44621-44627.

Deshimaru, M., Yoshimi, S., Shioi, S., Terada, S. (2004) Multigene family for Bowman-Birk type proteinase inhibitors of wild soya and soybean: the presence of two BBI-A genes and pseudogenes. *Bioscience, Biotechnology, and Biochemistry*, **68**, 1279-1286.

Douliez, J.P., Michon, T., Elmorjani, K., Marion, D. (2000). Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *Journal of Cereal Science*, **32**, 1-20.

Dunwell, J.M., Khuri, S., Gane, P.J. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiology and Molecular Biology Reviews*, **64**, 153-179.

Dunwell, J.M., Purvis, A., Khuri, S. (2004) Cupins: the most functionally diverse protein superfamily? *Phytochemistry*, **65**, 7-17.

Durán, Y. & Pérez de la Vega, M. (2004) Assessment of genetic variation and species relationships in collection of Lens using RAPD and ISSR. *Spanish Journal of Agricultural Research*, **2**, 538-544.

Echeverrigaray, S., Oliveira, A.C., Carvalho, M.T.V., Derbyshire, E. (1998) Evaluation of the relationship between lentil accessions using comparative electrophoresis of seed proteins. *Journal of Genetic and Breeding*, **52**, 89-94.

Erskine, W.(1997) Lessons for breeders from landraces of lentil. *Euphytica*, **93**, 107-112.

Gallo, G., Barbera, A.C., Venora, G. (1997) Valutazione agronomica di popolazioni di lenticchia (*Lens culinaris* Medik.) in ambienti semi-aridi. In: *Proceedings of the 3rd Convegno Nazionale sulla Biodiversità*, 16-17 June 1997, Reggio Calabria, Italy, 279-288.

Gökirmak, T., Paul, A.L., Ferl, R.J. (2010) Plant phosphopeptide-binding proteins as signaling mediators. *Current Opinion in Plant Biology*, **13**, 527-532.

Hu, X., Li, Y., Li, C., Yang, H., Wang, W., Lu, M. (2010) Characterization of Small Heat Shock Proteins Associated with Maize Tolerance to Combined Drought and Heat Stress. *Journal of Plant Growth Regulation*, **29**, 455-464.

Katam, R., Basha, S.M., Vasanthaiah, H.K.N., Naik, K.S.S. (2007) Identification of drought tolerant groundnut genotypes employing proteomics approach. *Journal of SAT Agricultural Research*, **5**, 1-4.

Kennedy, A.R. (1998) Chemopreventive agents: protease inhibitors. *Pharmacology and Therapeutics*, **78**, 167-209.

Ladizinsky, G. (1979) The origin of lentil and its wild gene pool. *Euphytica*, **28**, 179-187.

Ladizinsky, G. (1993) Wild lentils. *Critical Reviews in Plant Sciences*, **12**, 169-184.

Lambert, N. & Yarwood, J.N. (1992) Engineering legume seed storage proteins. In: *Plant Protein Engineering* (ed. P.R. Shewry & S. Gutteridge) London, UK.

Lázaro, A., Ruiz, M., de la Rosa, L., Martín, I. (2001) Relationships between agro/morphological characters and climatic parameters in Spanish landraces of lentil (*Lens culinaris* Medik.). *Genetic Resources and Crop Evolution*, **48**, 239-249.

Lechner, E., Achard, P., Vansiri, A., Potuschak, T., Genschik, P. (2006) F-box proteins everywhere. *Current Opinion in Plant Biology*, **9**, 631-638.

Link, A.J., Eng, J., Schieltz, D.M., Carmack, E., Mize, G.J., Morris, D.R., Garvik, B.M., Yates, J.R. (1999) Direct analysis of protein complexes using mass spectrometry. *Nature Biotechnology*, **17**, 676-682.

Ljung, K., Ostin, A., Lioussanne, L., Sandberg, G. (2001) Developmental regulation of indole-3-acetic acid turnover in Scots pine seedlings. *Plant Physiology*, **125**, 464-475.

Mello, M.O., Tanaka, A.S., Silva-Filho, M.C. (2003) Molecular evolution of Bowman-Birk type proteinase inhibitors in flowering plants. *Molecular Phylogenetics and Evolution*, **27**, 103-112.

Morrison, D.K. (2009) The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends in Cell Biology*, **19**, 16-23.

Mosolov, V.V. & Valueva, T.A. (2005) Proteinase inhibitors and their function in plants: a review. *Applied Biochemistry and Microbiology*, **41**, 227-246.

Oh, C.S. (2010) Characteristics of 14-3-3 proteins and their role in plant immunity. *Plant Pathology Journal*, **26**, 1-7.

Peng, J., Elias, J.E., Thoreen, C.C., Licklider, L.J., Gygi, S.P. (2003) Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: the yeast proteome. *Journal of Proteome Research*, **2**, 43-50.

Piergiovanni, A.R., Laghetti, G., Olita, G., Monti, M., Preiti, G., Di Prima, G. (1998) Screening for agronomic and biochemical traits in a lentil germplasm collection. In: *Proceedings of 3rd European Conference on Grain Legume*, 14-19 November 1998, Valladolid, Spain, 206.

Piergiovanni, A.R. (2000) The evolution of lentil (*Lens culinaris* Medik.) cultivation in Italy and its effects on the survival of autochthonous populations. *Genetic Resources* and *Crop Evolution*, **47**, 305-314.

Piergiovanni, AR. & Taranto, G. (2005) Assessment of the genetic variation in Italian lentil populations by electrophoresis (SDS-PAGE) of seed storage proteins. *Plant Genetic Resources Newsletter*, **141**, 33-38.

Poonam, T., Sharma, K., Mishra, S.K. (2006) Agro-morphological characterization of diverse germplasm of lentil (*Lens culinaris* Medik.). *New Botany*, **33**, 225-235.

Prakash, B., Selvaraj, S., Murthy, M.R., Sreerama, Y.N., Rao, D.R., Gowda, L.R. (1996)

Analysis of the amino acid sequences of plant Bowman-Birk inhibitors. *Journal of Molecular Evolution*, **42**, 560-569.

Qi, R.F., Song, Z.W., Chi, C.W. (2005) Structural features and molecular evolution of Bowman-Birk protease inhibitors and their potential application. *Acta Biochimica et Biophysica Sinica*, **37**, 283-292.

Rabilloud, T. (2000) *Proteome research: two-dimensional gel electrophoresis and identification methods (Principles and Practice)*, Springer, Berlin Heidelberg, Germany.

Ravet, K., Touraine, B., Boucherez, J., Briat, J.F., Gaymard, F., Cellier, F. (2009) Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. *The Plant Journal*, **57**, 400-412.

Roberts, M.R. (2003) 14-3-3 Proteins find new partners in plant cell signalling. *Trends in Plant Science*, **8**, 218-223.

Rocco, M., D'Ambrosio, C., Arena, S., Faurobert, M., Scaloni, A., Marra, M. (2006) Proteomic analysis of tomato fruits from two ecotypes during ripening. *Proteomics*, **6**, 3781-3791.

Rodríguez, M.M., Paredes, C.M., Bescerra, V.V. (1999) Isoenzymatic diversity in Chilean lentil germoplasm (*Lens culinaris* Medik.). *Agricoltura Técnica*, **59**, 186-195.

Roopashree, S., Singh, S.A., Gowda, L.R., Rao, A.G.A. (2006) Dual-function protein in plant defence: seed lectin from *Dolichos biflorus* (horse gram) exhibits lipoxygenase activity. *The Biochemical Journal*, **395**, 629-639.

Sales, M.P., Gerhardt, I.R., Grossi-De-Sá, M.F., Xavier-Filho, J. (2000) Do legume storage proteins play a role in defending seeds against bruchids? *Plant Physiology*, **124**, 515-522.

Scippa, G.S., Trupiano, D., Rocco, M., Viscosi, V., Di Michele, M., D'Andrea, A., Chiatante, D. (2008) An integrated approach to the characterization of two autochthonous lentil (*Lens culinaris*) landraces of Molise (south-central Italy). *Heredity*, 101, 136-144.

Scippa, G.S., Rocco, M., Ialicicco, M., Trupiano, D., Viscosi, V., Di Michele, M., Arena, S.,

Chiatante, D., Scaloni, A. (2010) The proteome of lentil (*Lens culinaris* Medik.) seeds: discriminating between landraces. *Electrophoresis*, **31**, 497-506.

Sehnke, P.C., Rosenquist, M., Alsterfjord, M., DeLille, J., Sommarin, M., Larsson, C., Ferl, R.J. (2002) Evolution and isoform specificity of plant 14-3-3 proteins. *Plant Molecular Biology*, **50**, 1011-1018.

Senatore, F., Schettino, O., Basso, F. (1992) Fatty acid and sterol content in eight populations of lentil (*Lens esculenta* Moench). *Rivista Italiana Sostanze Grasse*, **69**, 329-331.

Sharma, S.K., Dawson, I.K., Waugh, R. (1995) Relationships among cultivated and wild lentils revealed by RAPD analysis. *Theoretical and Applied Genetics*, **91**, 647-654.

Sharon, N. & Lis, H. (1990) Legume lectins-a large family of homologous proteins. *FASEB Journal*, **4**, 3198-3208.

Shewry, P.R., Napier, J.A., Tatham, A.S. (1995) Seed Storage Proteins: Structures and Biosynthesis. *The Plant Cell*, **7**, 945-956.

Sonnante, G. & Pignone, D. (2001) Assessment of genetic variation in a collection of lentil using molecular tools. *Euphytica*, **120**, 301-307.

Sonnante, G., De Paolis, A., Pignone, D. (2005) Bowman-Birk inhibitors in *Lens*: identification and characterization of two paralogous gene classes in cultivated lentil and wild relatives. *Theoretical and Applied Genetics*, **110**, 596-604.

Spencer, P.W. & Spencer, R.D. (1974) Globulin-specific proteolytic activity in germinating pumpkin seeds as detected by a fluorescence assay method. *Plant Physiology*, **54**, 925-930.

Spencer, D. (1984) The physiological role of storage proteins in seeds. *Philosophical Transactions of the Royal Society in London*, **304**, 275-785.

Sultana, T. & Ghafoor, A. (2008) Genetic diversity in *ex-situ* conserved *Lens culinaris* for botanical descriptors, biochemical and molecular markers and identification of landraces from indigenous genetic resources of Pakistan. *Journal of Integrative Plant Biology*, **50**, 484-490.

Talamo, F., D'Ambrosio, C., Arena, S., Del Vecchio, P., Ledda, L., Zehender, G., Ferrara, L., Scaloni, A. (2003). Proteins from bovine tissues and biological fluids: defining a reference electrophoresis map for liver, kidney, muscle, plasma and red blood cells. *Proteomics*, **3**, 440-460.

Thiellement, H., Bahrman, N., Damerval, C. (1999) Proteomics for genetic and physiological studies in plants. *Electrophoresis*, **20**, 2013-2026.

Toklu, F., Biçer, B.T., Karaköy, T. (2009) Agro-morphological characterization of the Turkish lentil landraces. *African Journal of Biotechnology*, **8**, 4121-4127.

Torricelli, R., Silveri, D.D, Ferradini, N., Venora, G., Veronesi, F., Russi, L. (2012) Characterization of the lentil landrace Santo Stefano di Sessanio from Abruzzo, Italy. *Genetic Resources and Crop Evolution*, **59**, 261-276.

Tullu, A., Kusmenoglu, I., McPhee, K.E., Muehlbauer, F.J. (2001) Characterization of core collection of lentil germplasm for phenology, morphology, seed and straw yields. *Genetic Resources and* Crop *Evolution*, **48**, 143-152.

Viscosi, V., Ialicicco, M., Rocco, M., Trupiano, D., Arena, S., Chiatante, D., Scaloni, A., Scippa, G.S. (2010) Lentils biodiversity: the characterization of two local landraces. In: *Tools for Identifying Biodiversity: Progress and Problems. Proceedings of the International Congress, Paris, September 20-22, 2010* (ed. P.L. Nimis & R.V. Lebbe), Trieste, EUT Edizioni Università di Trieste, 327-331.

Walz, A., Park, S., Slovin, J.P., Ludwig-Müller, J., Momonoki, Y.S., Cohen, J.D. (2002) A gene encoding a protein modified by the phytohormone indoleacetic acid. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 1718-1723.

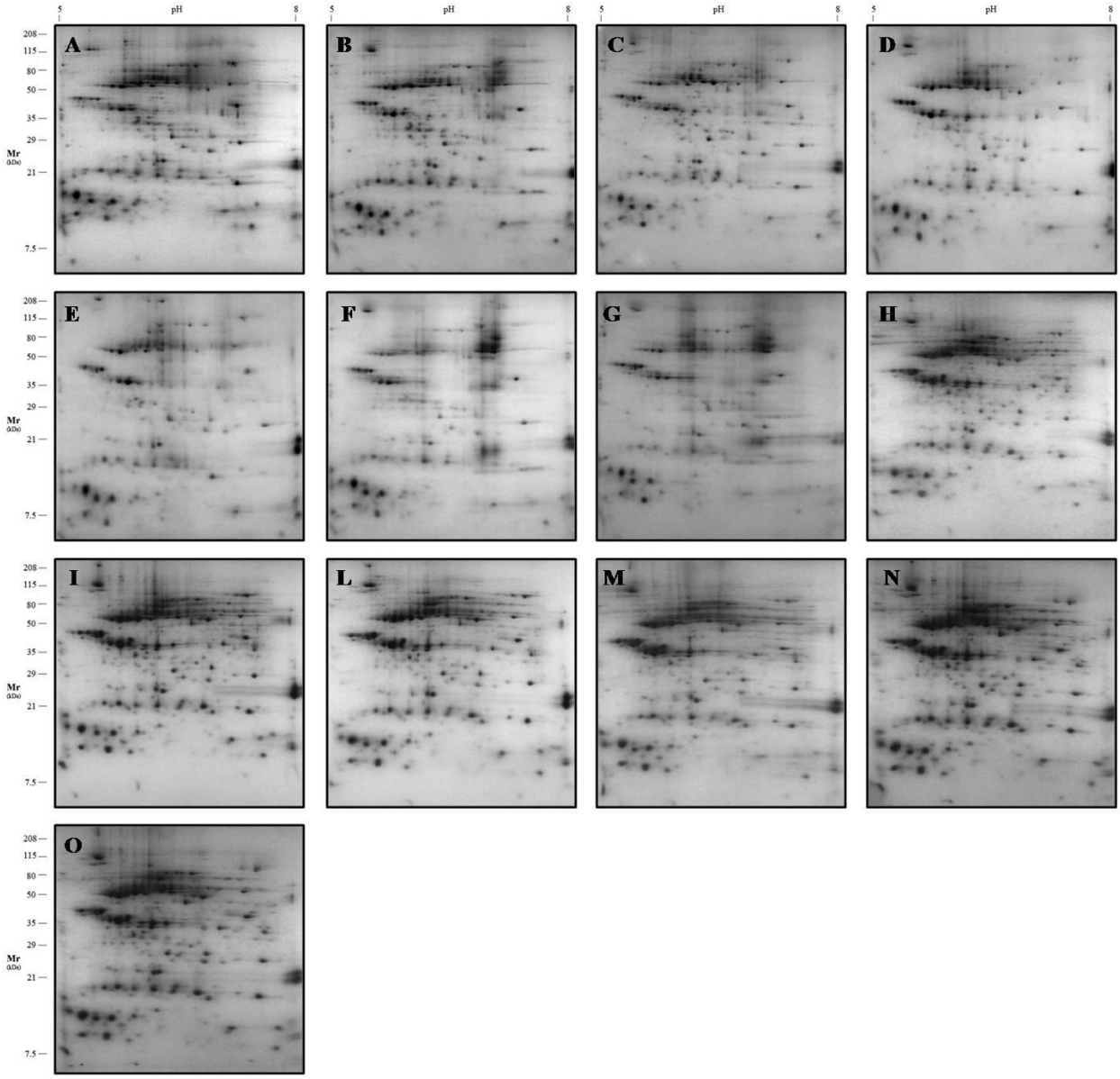
Zaccardelli, M., Lupo, F., Piergiovanni, A.R., Laghetti, G., Sonnante, G., Daminati, M.G., Sparvoli, F., Lioi, L. (2011) Characterization of Italian lentil (*Lens culinaris* Medik.) germplasm by agronomic traits, biochemical and molecular markers. *Genetic Resources and Crop Evolution*, 1-12.

Zohary, D. (1995) Lentil: *Lens culinaris* (Leguminoseae - Papilionoideae). In: *Evolution of Crop*

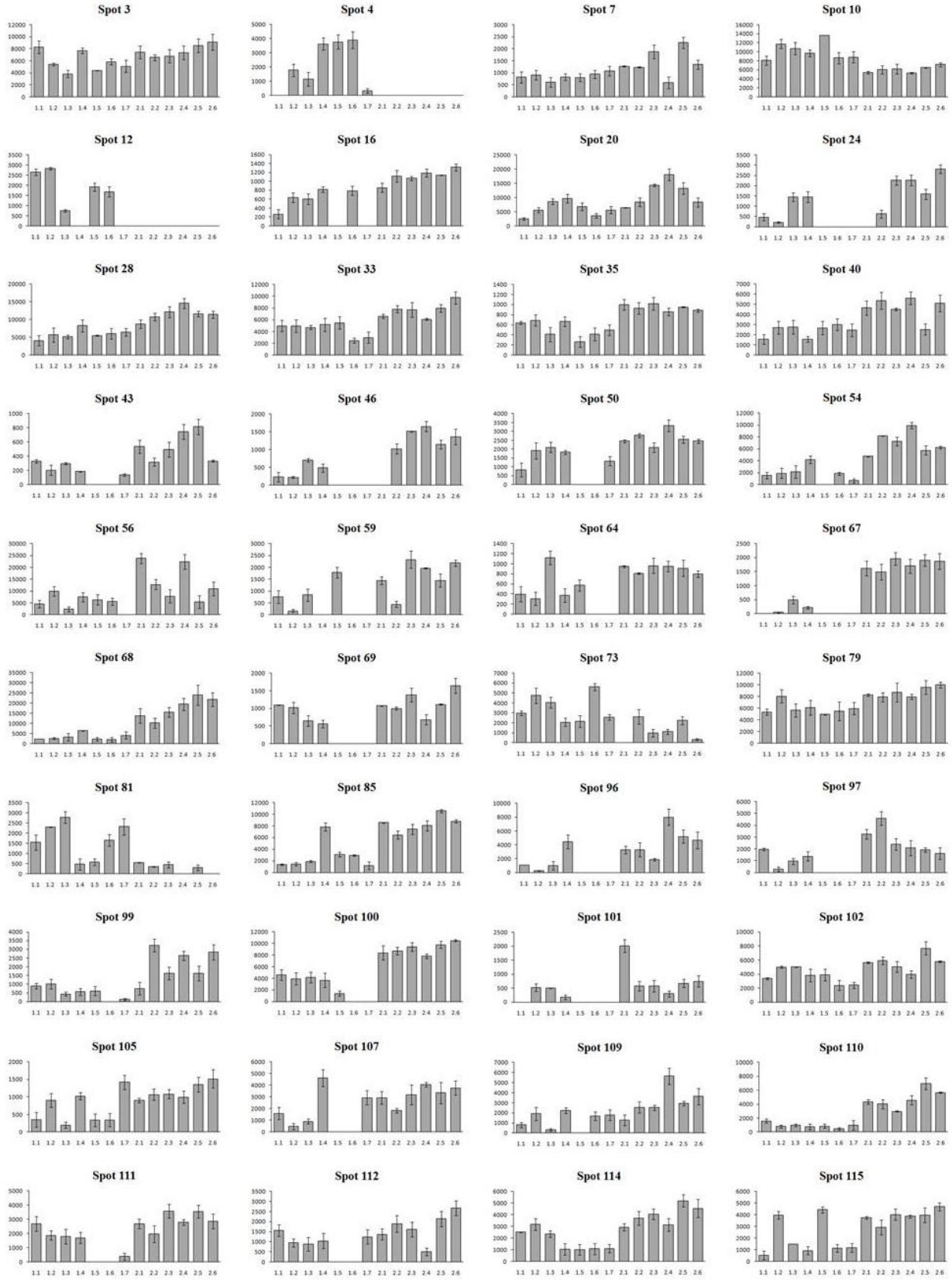
Plants (ed. J. Smartt & N.W. Simmonds) Longman Scientific & Technical. Harlow, UK., 271-274.

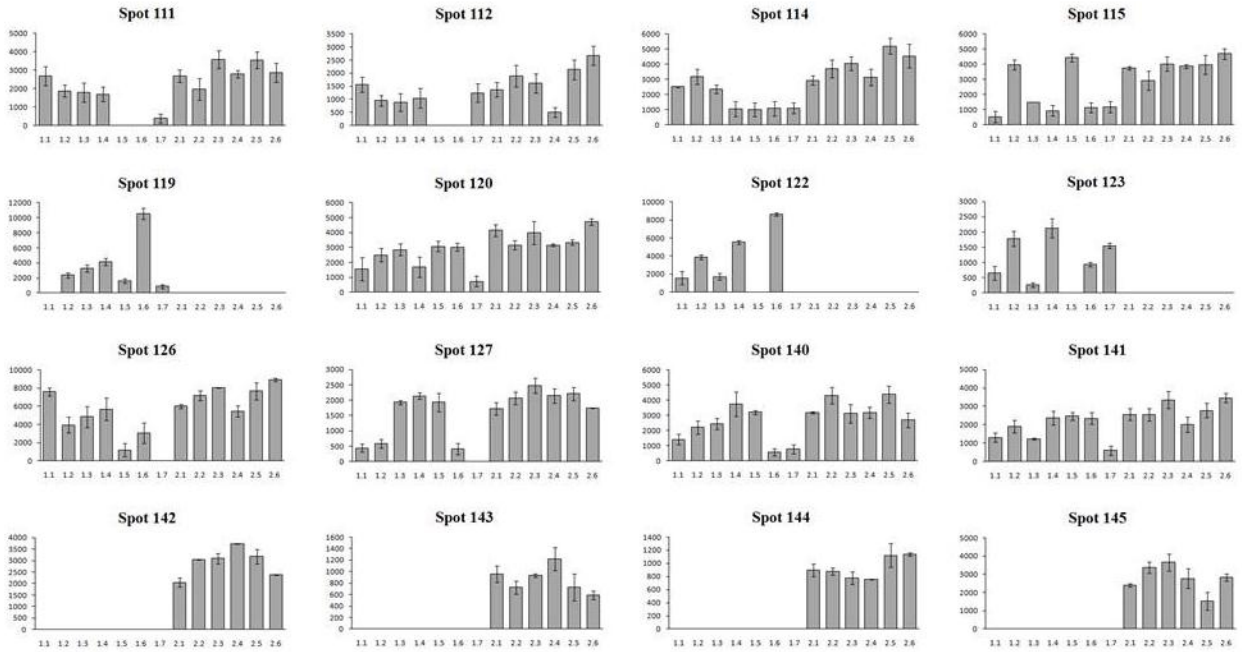
SUPPORTING INFORMATION

Supporting Information Figure S1. 2-DE analysis of the *Lens culinaris* dry mature seed populations. Gel profiles of total proteins from: A) Capracotta 1.1; B) Capracotta 1.2 (reference map); C) Capracotta 1.3; D) Capracotta 1.4; E) Capracotta 1.5; F) Capracotta 1.6; G) Capracotta 1.7; H) Conca Casale 2.1; I) Conca Casale 2.2; L) Conca Casale 2.3; M) Conca Casale 2.4; N) Conca Casale 2.5; O) Conca Casale 2.6. Gels were stained with colloidal CBB G-250. An equal amount (600 µg) of total protein extracts was loaded in each gel.



Supporting Information Figure S2. Differential expression of the 52 protein spots that resulted more indicative in discriminating Capracotta and Conca Casale lentil landraces, according to the ANOVA. Proteins were considered as differentially expressed when a relative fold change >2.0 or <0.5 was measured. 1.1-1.7 Capracotta; 2.1-2.6 Conca Casale.




Spot 111
Spot 112
Spot 114
Spot 115
Spot 119
Spot 120
Spot 122
Spot 123
Spot 126
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Spot 140
Spot 141
Spot 142
Spot 143
Spot 144
Spot 145

Supporting Information Table S1. This table shows F-ratios (F) and significance (Sig.) values for the 52 proteins that resulted significant in ANOVA for discriminating the Capracotta and Conca Casale lentil landraces.

Ref. Map ID	F	Sig.	Ref. Map ID	F	Sig.
67	253.773	0.000	96	10.873	0.007
144	221.502	0.000	73	10.659	0.008
142	165.443	0.000	123	10.099	0.009
143	103.295	0.000	64	9.853	0.009
145	96.750	0.000	102	9.475	0.011
100	52.871	0.000	4	9.132	0.012
110	50.924	0.000	59	8.930	0.012
68	47.158	0.000	126	8.627	0.014
28	40.230	0.000	12	8.473	0.014
35	38.274	0.000	141	8.410	0.014
54	35.773	0.000	69	8.377	0.015
79	28.296	0.000	115	8.006	0.016
85	25.271	0.000	109	7.874	0.017
33	23.584	0.001	56	7.692	0.018
10	22.954	0.001	20	7.468	0.019
40	21.530	0.001	127	6.727	0.025
114	19.568	0.001	101	6.641	0.026
16	18.462	0.001	7	6.579	0.026
99	17.307	0.002	140	6.482	0.027
43	15.950	0.002	112	5.777	0.035
97	14.236	0.003	105	5.698	0.036
81	13.850	0.003	3	5.628	0.037
50	13.728	0.003	122	5.477	0.039
46	12.494	0.005	119	5.140	0.045
120	12.483	0.005	24	4.898	0.049
111	11.983	0.005	107	4.861	0.050

df=1

Chapter II

Response of lentil (*Lens culinaris* Medik.) landraces to salt stress conditions.

This chapter reports the study concerning the "Response of lentil (*Lens culinaris* Medik.) landraces to salt stress conditions".

We evaluated the response of two lentil landraces from Molise (Capracotta and Conca Casale) and five widely spread commercial varieties (Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese) to salt stress using physiological and proteomic analysis. In particular, it has been investigated how salt stress affected seed germination and caused changes in protein expression. Furthermore, several proteins which may play an important role in saline stress response were identified.

INTRODUCTION

2.1 Environmental stresses

Being sessile, the plants are vulnerable to numerous environmental stresses, biotic (imposed by other organisms, e.g. pathogens, insects, herbivores) or abiotic (imposed by physical or chemical environmental changes, e.g. low and high temperatures, salinity, drought, excess water, radiation, heavy metals, wind), that can influence their growth, development and productivity (Mahajan & Tuteja, 2005).

Stress can be defined as a set of conditions that causes aberrant changes in physiological processes eventually resulting in injuries (Nilson & Orcutt, 1996).

Plant response to environmental stress is controlled by very complex mechanisms and involves alterations at different levels, depending on stress intensity, duration and severity and on plant genotype.

Plant responses to stress may be grouped in two general categories: avoidance, which prevents exposure to the stress, and tolerance, which permits the plant to withstand the stress (Levitt, 1972). Molecular factors controlling the avoidance and tolerance responses have been extensively investigated in many plants in relation to widely diffused abiotic stresses as drought, salinity and low temperature (Seki et al., 2003a; Seki et al., 2003b), and many transgenic plants have been produced with increased resistance (Wang et al., 2003; Zhang et al., 2004).

2.2 Types and causes of salinity

Salinity is the concentration of dissolved mineral salts present in the soils (soil solution) and waters. The dissolved mineral salts consist of the electrolytes of cations and anions. The major cations in saline soil solutions consist of Na^+, Ca^{2+}, Mg^{2+} and K^+ and the major anions are Cl^-, SO_4^{2-}, HCO_3^-, CO_3^{2-} and NO_3^- (Manchanda & Garg, 2008). Water soluble salts accumulate in the soil to a level that impacts on agricultural production, environmental health, and economic welfare (Rengasamy, 2006). The dominant sources of salt are rainfall and rock weathering. Rainfall contains low amounts of salt, but over time, salt deposited by rain can accumulate in the landscape. Wind-transported material from soil or lake surfaces is another source of salt. Poor quality irrigation water also contributes to salt accumulation in irrigated soils. The particular processes contributing salt, combined with the influence of other climatic and landscape features and the effects of human activities, determine where salt is likely to accumulate in the landscape (Rengasamy, 2006).

Salinity has been classified as ‘‘primary’’ or ‘‘secondary’’ salinity by Ghassemi et al. (1995). *Primary salinity* results from the accumulation of salts over long periods of time, from two natural processes. The first is the weathering of rocks containing soluble salts of various types,

mainly chlorides of sodium, calcium and magnesium, and to a lesser extent, sulfates and carbonates. The second is the deposition of oceanic salt carried inland by wind and rain. The salt composition of this deposited salt is that of seawater, that is, mainly sodium chloride (Munns, 2005).

Secondary salinity results from human activities that change the hydrologic balance in the soil between water applied (irrigation or rainfall) and water used by crops (transpiration). The most common causes are (1) land clearing and the replacement of perennial vegetation with annual crops, and (2) irrigation schemes using salt-rich irrigation water or having insufficient drainage.

2.3 Salt stress

Plant salt stress is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death. On a world scale, no toxic substance restricts plant growth more than does salt (Zhu, 2007).

Plants are stressed in two ways under high salt environmental conditions: by the decrease in osmotic potential of soil solution as a result of high solute content, and by the toxic effect of high concentration of ions (Demir & Kocakalikan, 2002).

First, salt in soil water inhibits plants ability to take up water, and this leads to slower growth. This is the osmotic or water-deficit effect of salinity (Munns, 2005). Water deficit under saline conditions is caused by low osmotic potentials of the soil solution. As a consequence, cell turgor pressure decreases. Since the growth of cells is correlated with turgor pressure in the growing tissues, decreased turgor is the major cause of inhibition of plant cell expansion under saline conditions (Greenway & Munns, 1980).

Second, high internal concentrations of Na^+ and Cl^- may provide toxic ions in the cellular compartment (Greenway & Munns, 1980), resulting in an inhibition of many physiological and biochemical processes such as nutrient uptake and assimilation (Hasegawa et al., 2000; Munns, 2002; Munns et al., 1995; Munns & Tester, 2008). This is salt specific or ion-excess effect of salinity (Munns, 2005). Plant growth is affected by interactions of Na^+ or Cl^- and many mineral nutrients, causing imbalances in the nutrient availability, uptake, or distribution within plants and also increasing the plant's requirement for essential elements (Greenway & Munns, 1980; Grattan & Grieve, 1992).

2.4 Effects of salinity on plants

Crop species show a spectrum of responses to salt, although all have their growth, and eventually, their yield reduced by salt. Salt responses are the combined result of the complex interaction among different morphological, physiological, and biochemical processes (Singh &

Chatrath, 2001). Morphological symptoms are indications of the injurious effects of salt stress. Salinity may directly or indirectly inhibit cell division and enlargement in the plant's growing point. Reduced shoot growth caused by salinity originates in growing tissues, not in mature photosynthetic tissues. As a result, leaves and stems of the affected plants appear stunted (Singh & Chatrath, 2001). Salt stress reduces dry matter content, increases root: shoot ratio, and diminishes leaf size; as a result grain yield is reduced (Singh & Chatrath, 2001).

Salt stress affects many aspects of plant metabolism and, as a result, growth is reduced. Photosynthesis is reduced because it is affected by leaf expansion rate, leaf area, and leaf duration, as well as by photosynthesis and respiration per unit leaf area. Mineral uptake by roots is affected as a result of imbalance in the availability of different ions. Proportions of Ca^{2+} in the medium that are adequate under non-saline conditions become inadequate under saline conditions. Growth inhibition by salt also occurs due to the diversion of energy from growth to maintenance (Nieman & Maas, 1978). The latter may include the regulation of ion concentration in various organs and within the cell, the synthesis of organic solutes for osmoregulation or protection of macromolecules, and for maintenance of membrane integrity (Jain et al., 2001). Osmoregulation ensures that adequate turgor is maintained in the cell. Organic compounds that accumulate in the cytoplasm may function as osmotica and in protecting the conformation of macromolecules in the changing ionic environment (Borowitzak, 1981; Wyn Jones & Pollard, 1983). Since salt damage has a broad physiological spectrum affecting many metabolic processes, it is difficult to access the contribution of individual processes to plant death or to the final damage done to the plant (Manchanda & Garg, 2008).

To overcome the saline stress plant cells tend to readjust their osmotic potential to prevent water losses that can be achieved either by uptake of inorganic ions from the external, or by de novo synthesis of compatible solutes. The osmotic readjustment is quickly more induced by the changes in ion fluxes than the synthesis of compatible solutes (Manchanda & Garg, 2008). According to Neumann et al. (1988), salt stress initially inhibits leaf expansion through reduced turgor and may in fact eventually result in increased cell wall extensibility, which counteracts the negative effects of low turgor. In the presence of salt, cell wall extensibility of the growing region may decrease (Cramer, 1992; Nonami et al., 1995). Gholipoor et al. (2000) observed a decrease in shoot water content with increasing salinity in four chickpea cultivars. Two chickpea cultivars, differing in drought tolerance, when grown under salinity had a slight effect on the leaf water potential, which were higher for the drought-tolerant variety (Katerji et al., 2001). Hernandez & Almansa (2002) reported that in pea leaves, salt stress brought about a decrease in osmotic potential and in stomatal conductance. Bayuelo-Jimenez et al. (2003) examined four

wild and two cultivated *Phaseolus* species grown under saline conditions and reported that salinity decreased the leaf water, osmotic and turgor potentials in all the species.

Salinity also decreases leaf water potential. Ahmad & Jhon (2005) reported decrease in the relative water content (RWC) in *Pisum sativum* L. leaves subject to salinity of 50, 100, 150 and 200 mM NaCl. Luo et al. (2005) studied differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *Glycine soja* under NaCl stress and reported that the control plants had higher osmotic potential (-0.05 MPa) than Cl, Na and NaCl-treated plants (-0.68 MPa).

Increases in ambient salt concentrations can lead to toxic accumulation of ions such as Cl^- and, in particular, Na^+ ion in the cytosol. The disproportionate presence of Na^+ in both cellular and extracellular compartments negatively impacts on the acquisition and homeostasis of essential nutrients such as K^+ and Ca^{2+}. The negative effects find their origin in many phenomena: ionic interactions between Ca^{2+} and cellular components such as cell wall pectins and membrane phospholipids are sensitive to excess cations such as Na^+. Although these sites typically have a much higher affinity for Ca^{2+} than for Na^+, the large molar Na^+: Ca^{2+} ratio leads to dissociation of Ca^{2+} from its binding sites, affecting the integrity of cell walls and cell membranes. In the cytosol, the presence of K^+ is essential for the activation of many enzymes, for example, those involved in pyruvate synthesis and protein translation. Due to physicochemical similarities between Na^+ and K^+, excess Na^+ tends to substitute K^+ for Na^+ at these binding sites and hence impair cellular biochemistry. A third mechanism affected by high levels of salinity is nutrient transport itself. Specific transport systems have been shown to be inhibited by the presence of large amounts of ions such as Cl^- and Na^+ (Manchanda & Garg, 2008).

Francois et al. (1990) found that an increase in substrate salinity was associated with a significant increase in the concentration of Cl, Na and Ca in the leaf tissue of cluster bean. On the other hand, leaf Mg, K and P concentrations were significantly reduced by increased soil salinity. Cordovilla et al. (1995a, b) analysed salinity effects on growth and nutrient composition in four grain legumes faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.), soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris* L.) and observed higher K levels in the roots than in the shoot and higher Na content in both roots and shoots.

Mamo et al. (1996) found chloride concentration in chickpea plant parts at salt levels (0-8 dS m^{-1}) two to five times that of Na with significant reduction in K^+ concentration. Bean plants when exposed to long-term salt effects showed very high leaf Cl^- concentrations and also an increase in Na^+ leaf concentrations (Sibole et al., 1998). Seeds of chickpea (*Cicer arietinum*) when germinated in the presence of NaCl showed a decrease in K content, while an increase in

Na content was observed which ultimately inhibited shoot and seed yield of all the cultivars (Sekeroglu et al., 1999). However, chickpea (*C. arietinum*) grown under saline conditions showed high yield despite of low Na and Cl concentrations in the tissue (Richter et al., 1999). Hernandez & Almansa (2002) examined the effect of short-term salt stress and recovery in pea plants and observed a linear increase in Na^+ concentration in salt treated plants; however, in the recovered plants, a slight reduction in the Na^+ concentration was observed. In soybean subjected to 40 mM NaCl, the Na content of the leaves, hypocotyls, and roots increased, whereas the K content remained unchanged (Sobhanian et al., 2010a).

Esechie et al. (2002) studied the effects of sodium chloride salinity on cation equilibrium in alfalfa (*Medicago sativa* L.) and observed that apart from Na, whose concentration increased significantly in all plant parts with increasing salinity, a decrease was observed in the concentration of P, K, Ca and Mg. *Phaseolus vulgaris* plants subjected to NaCl accumulated higher amounts of Cl^- in the leaves (Ashraf & Bashir, 2003). El-Hamdaoui et al. (2003) reported that salinity produced a decrease of B, Ca, K and Fe contents in the shoots of pea plants. While tissue concentrations of Cl^- and Na^+ ion increased significantly in response to salt treatment in four wild and two cultivated *Phaseolus* species, K^+ concentrations reduced (Bayuelo-Jimenez et al., 2003). Further, Ca^{2+} concentration of roots and stem remained approximately constant, whereas, leaf Ca^{2+} concentrations significantly increased with salinity.

Differential sensitivity to chloride and sodium ions in seedling of *Glycine max* and *Glycine soja* under NaCl stress was studied by Luo et al. (2005). Their results showed that Cl^- was more toxic than Na^+ to seedlings of *G. max*, the injury was positively correlated with the content of Cl^- in the leaves, and negatively with that in the roots.

Morphologically, the most typical symptom of saline injury to a plant is retarded growth due to inhibition of cell elongation (Nieman, 1965). According to Okusanya & Ungar (1984), the roots are among the most sensitive organs to salinity. However, in their reports, Munns & Termaat (1986) suggested that, under increased salinity, root growth is almost always less affected than shoot growth. Elsheikh & Wood (1990) reported that salinity significantly reduced the dry weights of both shoots and roots in chickpea (*C. arietinum* L.). Delgado et al. (1994) and Cordovilla et al. (1995a, 1995b) compared the effect of salinity on growth and productivity and found pea and faba bean to be significantly affected and soybean and common bean to be moderately affected.

Soussi et al. (1998, 1999) reported decline in plant growth of *C. arietinum* L. by salt. Growth inhibition by salt in common bean plants proved significant in the experiments carried out by Ferri et al. (2000). Serraj et al. (2001) studied the short-term effects of NaCl on growth of four genotypes of common bean. After 5 days exposure to stress, the shoot dry weight of two

genotypes, W and B was 83% of that of control plants not exposed to NaCl with no significant effect of the salt treatment on the growth of other two genotypes, R and C. Saadallah et al. (2001) studied genotypic variability for tolerance to salinity of a common bean lines and reported that except for BAT477, salt significantly decreased the growth of all other lines.

Salinity-induced dry matter reduction are more severe in the shoot than the root as reported in soybean (Bernstein & Ogata, 1966; Shalhevet et al., 1995) and chickpea (Lauter et al., 1980). Welfare et al. (2002) studied the effect of saline environment on two chickpea cultivars and observed a substantial reduction in plant height, number of leaves and dry weights of leaves, stems and roots. Salinity inhibited shoot growth more than root growth, increased root:shoot ratio, decreased the number of leaves, leaf area in four wild and two cultivated *Phaseolus* species (Bayuelo-Jimenez et al., 2003). Ashraf & Bashir (2003) have reported significant reduction in fresh and dry weights of shoots and roots, and shoot length and leaf area of *P. vulgaris* under salt stress. Gama et al. (2007) examined the response of five cultivars of common bean to salt stress, and observed that salinity had adverse effects on plant height, number of leaves, root length and shoot/root weight ratio.

Bandeoglu et al. (2004) studied the growth performance of lentil to NaCl-salinity stress and reported significant reduction in shoot-root length and fresh-dry weights of the plants. A reduced plant growth was also reported by Luo et al. (2005) in seedlings of *Glycine max* and *Glycine soja* under NaCl stress.

Singla & Garg (2005) studied the influence of salinity on growth and yield attributes in chickpea cultivars and concluded that root growth was more adversely affected than shoot growth; which also had an impact on the root to shoot ratio and plant productivity. Jebara et al. (2005) reported negative effects of salt stress on plant growth in common bean plants. Singh et al. (2005) analysed ten genetically diverse chickpea lines for salt tolerance. They reported a decline in shoot, root and the single plant weight with increasing level of salt.

Salinity can affect germination of seeds either by creating osmotic potential which retard or prevent water uptake, or by toxic effects of ions on embryo. Several studies indicated that salt stress influences negatively germination and emergence (Kuruvadi, 1988; Ashraf & McNeilly, 1989; Mossad et al., 1992; Al-Mutawa, 2003; Jamil et al., 2005); in particular, the salinity at lower level delays the germination and at higher level reduces the germination (Kuhad & Sheoran, 1987; Jamil & Rha, 2004). The rate and percentage of seed germination of chickpea were significantly reduced by increasing salinity levels (Sekeroglu et al., 1999; Kafi & Goldani, 2001; Al-Mutawa, 2003). Kaymakanova (2009) reported decrease in the percentage of germination and seedlings growth of three bean cultivars (*Phaseolus vulgaris* L.) when subjected to salt stress. Effect of salinity on germination of lentils were investigated by Mohammad et al.

(2011), who found decreased germination with increasing concentration of NaCl solution. Dell'Aquila (2004) observed a gradual reduction of germination of lentil seeds after 16, 40 and 64 h exposure to 0.33 M NaCl.

Salinity promotes the synthesis of salt stress-specific proteins (Hurkman & Tanaka, 1987; Ben-Hayyim et al., 1989; Artlip & Funkhouser, 1995; Mahmoodzadeh, 2009), and causes either decreases (Popova et al., 1995) or increases (Dubey, 1983; Elenany, 1997) in the level of total and/or soluble proteins, depending on the plant parts studied.

In many cases increased protein levels are observed under salinization in germinating seeds (Dubey & Rani, 1987), growing seedlings (Dubey & Rani, 1989), and different plant parts (Elsamad & Shaddad, 1997). Under increasing levels of NaCl salinity, rice seedlings show an increased level of total as well as soluble proteins compared with non-stressed seedlings (Dubey & Rani, 1989). Similar to rice, the NaCl salinity treatment caused an increase in the protein content in cowpea (*Vigna unuiculata* L.) seedlings, pea (*Pisum sativum* L.), and beans (*Phaseolus vulgaris* L.) plants (Mehta & Vora, 1987; Vyas & Rao, 1987; Dantas et al., 2007).

Ashraf & Bashir (2003) reported that soluble proteins declined in all plant parts of *P. vulgaris* under salt stress (3.5 dS m^{-1} of NaCl). In chickpea (*Cicer arietinum* L.), a salinity treatment with 100 mM NaCl caused a marked decrease in the level of proteins in developing seeds (Murumkar & Chavan, 1986). When rice (*Oryza sativa* L.) seeds were germinated under increasing levels of NaCl salinity, a decrease in the total as well as the soluble protein level was observed in the embryo axes (Dubey & Rani, 1987). In barley plants, the imposition of NaCl stress leads to a decrease in the leaf protein content and induces marked quantitative and qualitative changes in the polypeptide profiles (Popova et al., 1995). Salinity reduced the protein contents of leaves in two chickpea cultivars (Sharma et al., 1990). In lentil, Ashraf & Waheed (1993) reported that leaf soluble proteins decreased due to salt stress in all lines, irrespective of their salt tolerance.

Garg et al. (1998) reported that salinity induced changes in levels of leaf soluble protein in cluster bean. Sousa et al. (2003), studied the protein pattern in cowpea seedlings subjected to salinity (100 mM NaCl) by isolating the albumins from stems and leaves and reported that in stem, 19 (14.6-76.3 kDa) proteins had their relative concentration increased by salinity, 8 (31.2-5.0 kDa) had their relative concentration decreased by salinity and 9 (16.3-39.8 kDa) were apparently synthesized de novo, while in the leaves, 9 (18.2-33.2 kDa) proteins increased in concentration, one (17.1 kDa) decreased in concentration and one (21.2 kDa) was apparently synthesized de novo.

Several salt-induced proteins have been identified in plants species and have been classified into two distinct groups (Hurkman et al., 1989; Pareek et al., 1997; Ali et al., 1999; Mansour,

2000); salt-stress proteins, which accumulate only due to salt stress, and stress-associated proteins, which also accumulate in response to heat, cold, drought, water-logging, and high and low mineral nutrients (Ashraf & Harris, 2004). Proteins may be synthesized de novo in response to salt stress or may be present constitutively at low concentration and increase when plants are exposed to salt stress (Pareek et al., 1997).

Many researchers had used proteomic approach for the identification of salt-responsive proteins in several plants. Salekdeh et al. (2002a) identified several salt-responsive proteins in root proteome of salt-tolerant and salt-sensitive rice varieties, including ABA- and stress-responsive proteins, ascorbate peroxidase, and many others. Several proteins were found to be modulated in expression by salt concentration in a coordinated manner (Nohzadeh et al., 2007). These proteins were involved in photosynthesis, photorespiration, signal trasduction, metabolism regulation, oxidative stress defence, control of ion channels, and protein folding. Two-dimensional electrophoresis was used also by Dooki et al. (2006) to reveal changes in protein expression of rice in response to salt stress at seedling stage; they identified several salt responsive proteins including an ABA- and stress-responsive protein, ascorbate peroxidase, and caffeoyl-CoA O-methyltransferase. Kav et al. (2004) studied the proteome-level changes in the roots of *Pisum sativum* in response to salinity and reported reproducible abundance in root protein extracts from whole plants and seedlings. These were identified as pathogenesis-related (PR) 10 proteins, antioxidant enzymes such as superoxide dismutase as well as nucleoside diphosphate kinase. The proteome of soybean subjected to salinity has been analyzed using roots and hypocotyls of young seedlings (Aghaei et al., 2009) and using different tissues (Sobhanian et al., 2010a). In particular, Aghaei et al. (2009) reported that seven proteins were found to be up- or down-regulated in response to salt stress: late embryogenesis-abundant protein, β-conglycinin, elicitor peptide three precursor, and basic/helix-loop-helix protein were up-regulated, while protease inhibitor, lectin, and stem 31-kDa glycoprotein precursor were down-regulated. Sobhanian et al. (2010a) found that 90, 22 and 14 proteins in the leaves, hypocotyls and roots, respectively, were up- and down-regulated by NaCl treatment. Metabolic-related proteins in the leaves, hypocotyls, and roots of soybean are mainly down-regulated under salt stress. Glyceraldehyde-3-phosphate dehydrogenase was down-regulated in the leaf/hypocotyls, and fructokinase 2 was down-regulated in the hypocotyls/root by salt stress. Stem 31 kDa glycoprotein precursor was up-regulated in all three organs with NaCl treatment. Glyceraldehyde-3-phosphate dehydrogenase was specifically down-regulated in the presence of salinity. Dell'Aquila (2004) studied the modifications in the protein patterns of lentil embryo axes under salt stress and observed a significant increase in abundance of proteins comprised

between the 5.1-7.6 pH isoelectric point in the first dimension and 75-50 kDa molecular mass in the second dimension.

2.5 Aim of the study

Italy's lentil cultivation is mostly based on landraces, local varieties empirically selected by farmers over time, well adapted to the environmental conditions in which they have been grown for decades and characterized by a wide genetic variability. In the recent past, its cultivation has progressively been reduced and, consequently, many local populations were disappeared and those still being cultivated are at a high risk of genetic erosion. The main reasons for the disappearance of lentil local populations are the progressive neglect of the marginal areas, the farmers' acculturation and the switch to more remunerative crops. There is an urgent need to promote the survival of lentil landraces at high risk of genetic erosion in order to avoid the irreversible loss of genes that may be used in breeding programs. In a previous study, we have assessed the genetic relationship between lentil landraces from Capracotta and Conca Casale, two little villages of Molise, and five commercial varieties using genomic, morphological and proteomic analysis. We found that the two Molise lentil landraces were significantly differentiated from each other; in particular, Conca Casale was well separated from commercial varieties, showing homogeneity at morphological, genetic and biochemical level, whereas Capracotta was separated from commercial varieties at genetic level with the exception of Castelluccio di Norcia, but was characterized by morphological and biochemical heterogeneity. In further study, we studied the relationship between lentil landrace from Capracotta and commercial varieties using a proteomic approach. We observed that all Capracotta populations were newly distinguishable from the commercial varieties, except Castelluccio di Norcia; in addition, we found 24 specific protein markers which were essential to discriminate the different lentil populations. In particular, Capracotta landrace was characterized by different levels of protein involved mainly in environmental stress response.

On the basis of the results obtained in the studies previously carried out, this work was undertaken to evaluate if Capracotta and Conca Casale lentil landraces, characterized by high genetic diversity and rusticity, responded better to abiotic stresses than commonly marketed lentil varieties (Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese). To accomplish this objective, we chose to analyze both physiological and biochemical response of lentil seed to salt stress and compared it with that of commercial lentil varieties.

Germination tests previously performed in presence of salt solutions showed that 10b, 14 and 17 Capracotta specimens and 28, 30 and 36 Conca Casale samples had higher germination percentage among already studied seven and six populations from Capracotta and from Conca

Casale, respectively. All lentil populations that get analyzed in this study belong to microsperma group with exception of 10b sample which is macrosperma.

2.6 Materials and methods

2.6.1 Plant materials

Molise lentil landraces (three from Capracotta and three from Conca Casale - two small villages in the Molise region of central-south Italy) and five others (Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese) were used in this study. In particular, Castelluccio di Norcia and Colfiorito are two traditional local populations of Umbria (Central Italy), Rascino is from other area of Central Italy (Lazio region), whereas Turca Rossa and Canadese are commercial varieties widely marketed.

L. culinaris Medik. seeds of all autochthonous landraces and commercial varieties were provided by the Molise Germoplasm Bank at the University of Molise (Pesche).

2.6.2 Germination test

Prior to germination, the seeds of each lentil population were surface sterilized in a solution of 4% sodium hypochlorite for 30 min to prevent fungal attack and rinsed in distilled water. The seeds were germinated in 9 cm diameter Petri dishes on two Whatman filter papers moistened with distilled water (control) or 100mM and 200mM NaCl solution. All Petri dishes were subsequently kept at a constant temperature 22°C and a 16 h light: 8 h dark photocycle. All solutions were renewed every three days. Germination test was performed on three replicates of 20 lentils seeds for each treatment.

The number of seeds germinated were counted daily and data were recorded for 16 days. A seed was considered germinated when the radicle emerged from the coat.

The results are expressed as final germination percentage (G%) and germination speed, expressed as number of days required to reach 50% germination (T_{50}) according to Bacchetta et al. (2006).

2.6.3 Protein extraction

For total protein extraction, 0.20 g of the control and of 200 mM NaCl treated samples were reduced to powder by mechanical breakage with liquid nitrogen and suspended in 5ml of 10% trichloroacetic acid, 0.07% β-mercaptoethanol and 1mM PMSF in cold acetone at -20°C (Rabilloud, 2000). The mixed sample was then filtered through Miracloth and proteins were precipitated overnight at -20°C. Each sample was centrifuged at 35,000 x g for 15 min at 4°C, the protein pellet was recovered, resuspended in 5 ml of 0.07% β-mercaptoethanol in cold

acetone and precipitated overnight at -20°C. The precipitated proteins were recovered by centrifugation at 35,000 x g for 15 min at 4°C, vacuum dried and solubilized in 300 µl of 7M urea, 2M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 20mM Tris-HCl, 1% (w/v) DTT, containing 0.2% (w/v) ampholine 3-10 and 0.15% (w/v) ampholine 5-7. Protein concentration was determined by the Bradford assay (Bradford, 1976) using a SmartSpec Plus Spectophotometer (BioRad) and BSA as standard.

2.6.4 Two-dimensional electrophoresis

For isoelettrofocusing (IEF), 700 µg protein samples were loaded on 17 cm ReadyStrip IPG strips with 5-8 linear pH gradient range (BioRad). Strips were rehydrated overnight at room temperature and IEF was performed with the PROTEAN IEF Cell system (BioRad) at 12°C, using a 50 µA current limit per strip and a program setting of 90 min at 250V, 90 min at 500V, 180 min at 1000V and 8000V for a total of 55 kVh (Rocco et al., 2006).

Prior to the second dimension, the IPG strips were equilibrated for 20 min in the first equilibration buffer containing 50mM Tris-HCl pH 8.8, 6M urea, 30% (w/v) glycerol, 2% (w/v) sodium dodecyl sulphate and 1% (w/v) DTT, followed by the second equilibration buffer in which DTT was substituted by 2.5% (w/v) iodoacetamide. The second dimensional separations were carried out on 12% SDS-polyacrylamide gels (18cm x 24cm x 1mm) on a Protean apparatus (BioRad) in 25mM Tris-HCl pH 8.3, 1.92M glycine and 1% (w/v) SDS, at 120V for about 16 h, until the bromophenol blue dye front reached the bottom of the gel. To visualize the protein spots, two-dimensional electrophoretic gels were stained with colloidal Coomassie G250. Each sample was run in triplicate.

2.6.5 Gel image analysis

Coomassie stained gels were scanned with a GS-800 calibrated densitometer (BioRad) and processed using PDQuest software (BioRad) that allowed performing a comparative image analysis. Automatic detection and matching of spots were carried out, followed by manual verification to check undetected or incorrectly matched spots. The gel used as reference in the PDQuest matchset corresponded to Conca Casale 36 control sample, and was subsequently used to excise spots for protein identification.

For each gel, normalized spot quantity was calculated as the ratio of each spot density to the whole-gel densities. The percentage volume of each spot was averaged for three different replicates of each biological sample, and Student's *t* test ($P<0.01$) was used to identify significant changes in protein folding between samples. A two-fold change in normalized spot densities was considered indicative of differences in expression levels.

The molecular masses of protein spots on gels were calculated by standard protein markers (BioRad).

2.6.6 In gel protein digestion and MS analysis

Spots from 2-DE were manually excised from the gel, triturated and washed with water. Proteins were in-gel reduced, S-alkylated and digested with trypsin, as reported by Talamo et al. (2003). Protein digests were subjected to a desalting/concentration step on μZipTipC18 pipet tips (Millipore) before MALDI-TOF-MS analysis and/or nanoLC-ESI-LIT-MS/MS analysis.

For MALDI-TOF peptide mass fingerprinting (PMF) experiments, peptide mixtures were loaded on the instrument target together with CHCA as matrix, using the dried droplet technique. Samples were analyzed with a Voyager-DE PRO spectrometer (Applied Biosystems). Peptide mass spectra were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. Data were elaborated using the DataExplorer 5.1 software (Applied Biosystems).

For nanoLC-ESI-LIT-MS/MS experiments, peptide mixtures were analyzed using a LTQ XL mass spectrometer (Thermo) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon). Peptide mixtures were separated on an Easy C18 column (10 x 0.075 mm, 3 μm; Proxeon) using a linear gradient from 5% to 50% of acetonitrile in 0.1% formic acid, over 60 min, at a flow rate of 300 nL/min. Spectra were acquired in the range m/z 300-1800. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 3 min). The mass isolation window and collision energy were set to m/z 3 and 35%, respectively.

2.6.7 Protein identification

MASCOT software package was used to identify spots unambiguously from NCBI updated plant non-redundant sequence database. MALDI-TOF PMF data were searched using a mass tolerance value of 40-80 ppm, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. NanoLC-ESI-LIT-MS/MS data were searched by using a mass tolerance value of 2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively.

MALDI-TOF PMF candidates with a cumulative MASCOT score≥58 or nanoLC-ESI- LIT-MS/MS candidates with more than 2 assigned peptides with an individual MASCOT score≥25, both corresponding to $p<0.05$ for a significant identification, were further evaluated by the

comparison with their calculated mass and pI values, using the experimental values obtained from 2-DE.

2.6.8 Statistical analysis

For each protein resulted as differentially expressed by PDQuest analysis, the quantitative data were subjected to uni- and multivariate analysis.

For analysis of variance (ANOVA), proteins with P value ≤ 0.05 were considered statistically significant to detect changes in the quantities of individual protein spot between control and 200 mM NaCl treated samples.

Cluster analysis was carried out on protein spots resulted significant by ANOVA using a correlation matrix and paired group as linkage in order to group the proteins according to their expression profile; a phenogram was built-up, and bootstrap values were computed on 999 random permutations.

2.7 Results

2.7.1 Germination test

The effects of different concentrations of NaCl solution on final germination percentage (G%) and germination speed (T_{50}) of three populations of lentil from Capracotta are showed in Figure 1.

In detail, the three lentil populations had different final germination percentages and T_{50} values in control conditions. In particular, Capracotta 10b sample showed the lowest percentage of germination (73%) and the highest T_{50} (2.6 days), whereas Capracotta 14 and 17 specimens had high G% value, namely, 97% with T_{50} of 1.6 and 1.4 days, respectively (Figure 1). Under salt stress, a reduction of percentage of germination and an increase of T_{50} were observed for all Capracotta lentil seeds. As shown in Figure 1, raising the salt concentration the germination percentage decreased in Capracotta 14 and 17. However, an exception was represented by Capracotta 10b population for which there was a slight increase in G% (78%) when seeds were germinated in 100 mM NaCl solution. At 200 mM NaCl, germination percentage was significantly reduced in Capracotta 10b (33%) and 14 (50%), while slightly affected in Capracotta 17 with a percentage of 92% (Figure 1) at the same salinity level (200 mM NaCl).

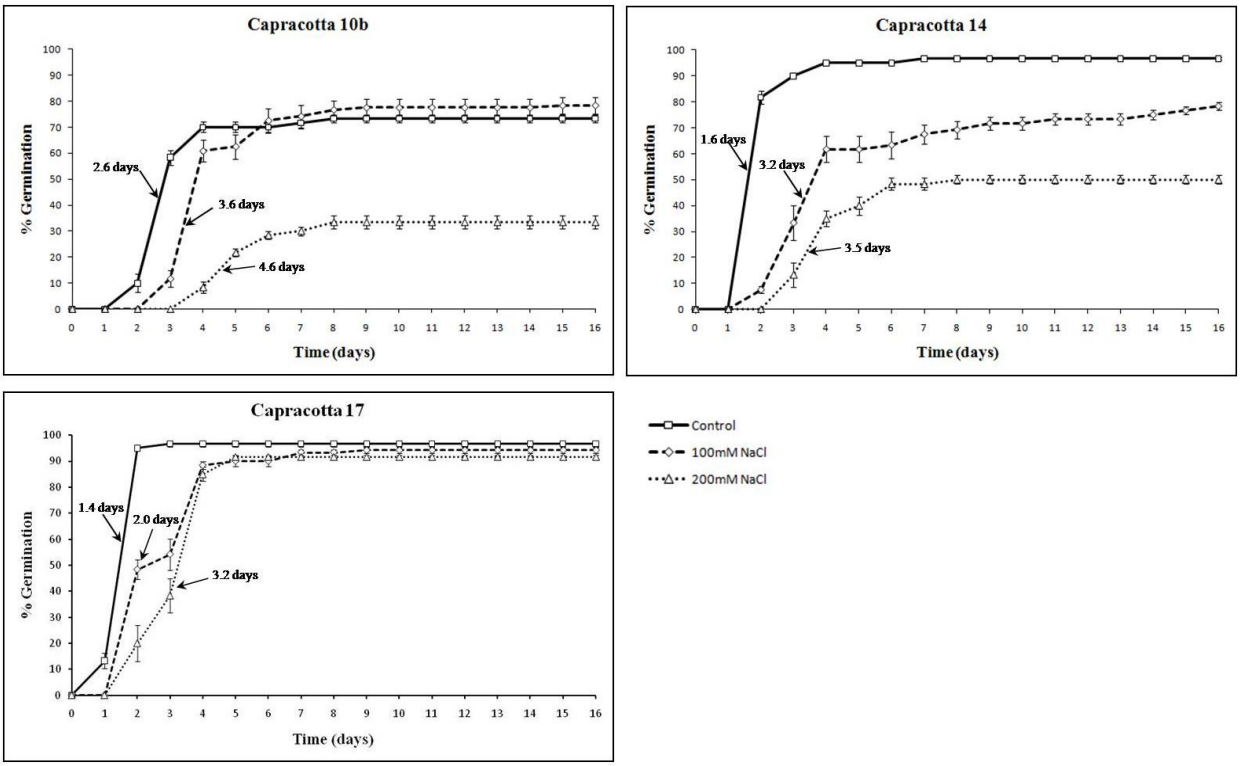

Figure 1. Germination of Capracotta lentil seeds in distillated water (control), in 100 mM and 200 mM NaCl solution (stress). Arrows indicate mean germination time for each treatment; vertical bars represent the standard deviation of the mean of three replicates of 20 seeds each.

The results of seed germination of three lentil populations from Conca Casale are presented in Figure 2.

Under control conditions, Conca Casale 28, 30 and 36 seeds had very similar germination percentages rangingbetween 95% and 98% (Figure 2). When exposed to 100 mM NaCl, T_{50} increased for all specimens, instead the final germination was slightly reduced in Conca Casale 30 and 36 samples (89% and 88%, respectively), whereas it decreased markedly (48%) in Conca Casale 28 (Figure 2). The percentage of germination of Conca Casale 28 and 30 was adversely affected by 200 mM NaCl (40% and 68%, respectively), whereas it remained high for Conca Casale 36, namely, 85% (Figure 2). In addition, germination speed was reduced for all populations when seeds germinated in presence of 200 mM NaCl solution; in fact, T_{50} values increased compared with those of the control and 100 mM NaCl treatment (Figure 2).

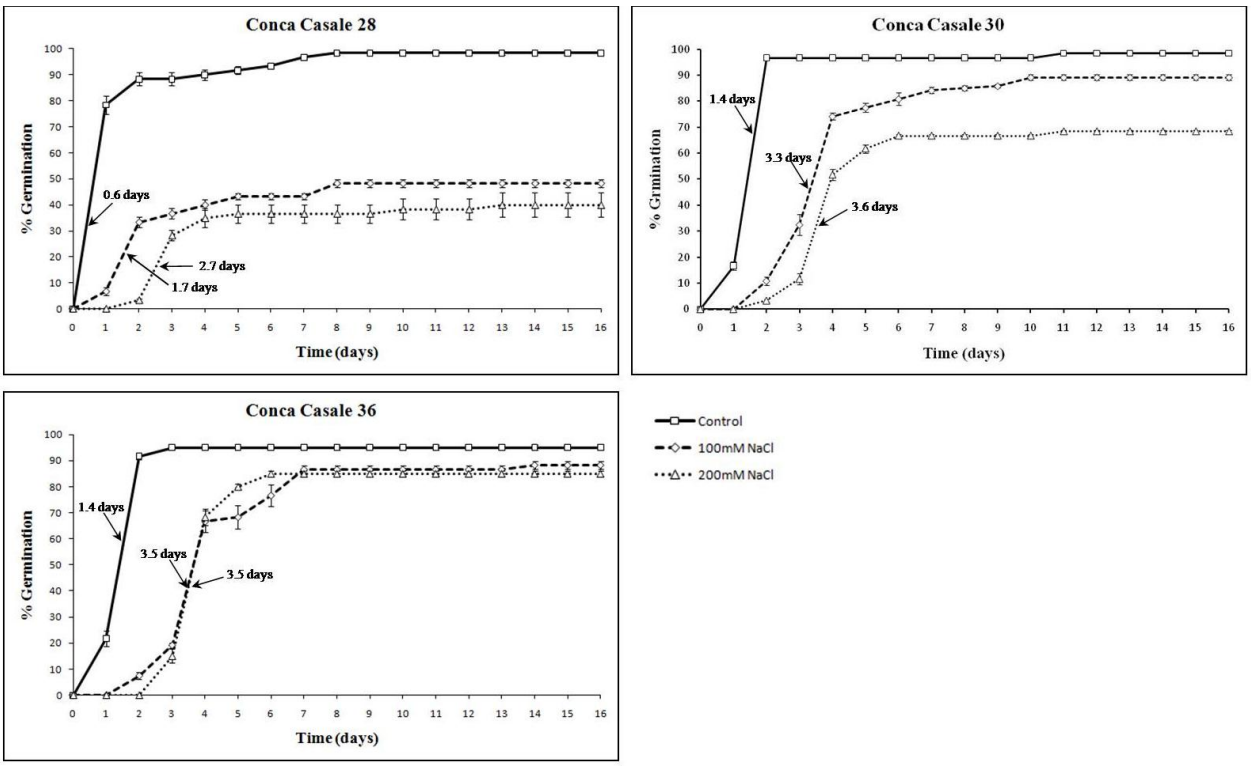

Figure 2. Germination of Conca Casale lentil seeds in distillated water (control), in 100 mM and 200 mM NaCl solution (stress). Arrows indicate mean germination time for each treatment; vertical bars represent the standard deviation of the mean of three replicates of 20 seeds each.

Figure 3 shows the results of germination test for Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese lentil seeds.

Control seeds of the five varieties had similar final germination percentages that ranged from 80% for Rascino sample to 87% for Colfiorito sample, except for Castelluccio di Norcia which showed a lower value of G%, namely, 68% (Figure 3). When seeds were exposed to 100 mM NaCl, final germination percentage decreased to 63% for Colfiorito and to 77% for Rascino, whereas Castelluccio di Norcia, Turca Rossa and Canadese varieties increased slightly their G% (69%, 90% and 88%, respectively), as shown in Figure 3. The highest salt stress condition (200 mM NaCl) caused a strong decrease in germination percentage of Castelluccio di Norcia (32%), Colfiorito (23%) and Rascino (47%), while a minor reduction was observed in Turca Rossa (67%) and Canadese (68%) varieties (Figure3). Furthermore, T_{50} increased when the salinity level was raised in all five lentil commercial varieties (Figure 3).

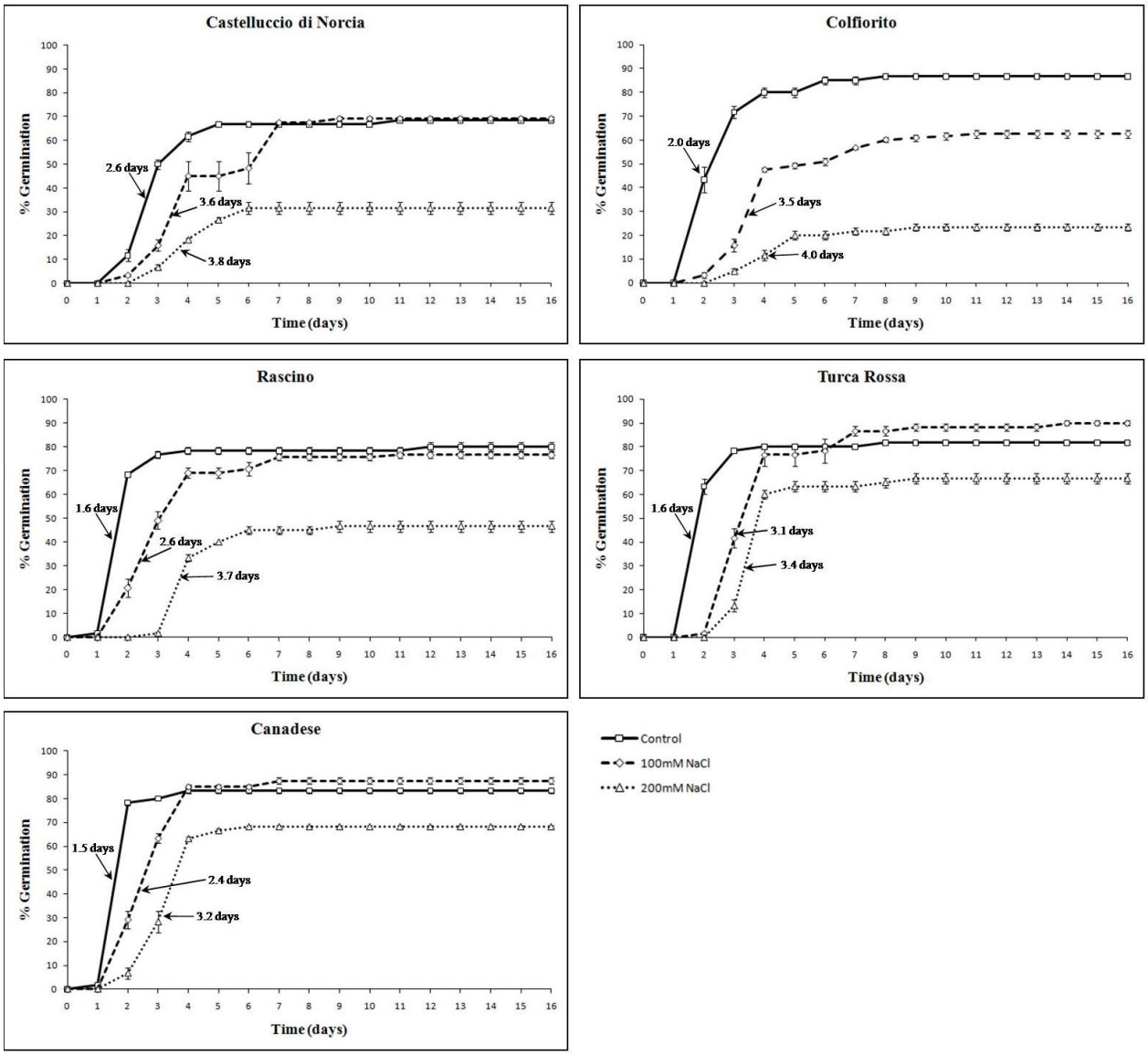

Figure 3. Germination of Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese lentil seeds in distillated water (control), in 100 mM and 200 mM NaCl solution (stress). Arrows indicate mean germination time for each treatment; vertical bars represent the standard deviation of the mean of three replicates of 20 seeds each.

In general the results obtained showed that salt treatment had an adverse effect on total germination percentage and rate of germination in all studied lentil populations; in particular, 100 mM NaCl solution decreased mainly the speed of germination, whereas final percentage of germination was markedly suppressed at the highest level (200 mM) of NaCl.

Given that 200 mM NaCl more significantly affected lentil seed germination, this concentration of NaCl was chosen for further analysis.

2.7.2 Comparative proteome analysis

To detect the proteins variably expressed in response to salt stress, 2-DE pattern of germinated lentil seeds from control and 200 mM NaCl treatment were compared. Total proteins

from control and stressed seeds of all 11 lentil populations (three from Capracotta, three from Conca Casale, Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese) analysed in this study were extracted and separated by two-dimensional electrophoresis. The 2-DE maps, obtained using a linear 5-8 pH gradient, showed a high level of reproducibility and were well-resolved with an average of 500-550 distinguishable spots distributed between molecular masses of 5-121 kDa (Figura 4, Figure 5). In addition, the majority of spots detected in these maps had analogous positions, as indicated by the degree of gel similarity (56%-87%) between the different specimens and the reference map (Conca Casale 36 control sample) which was characterized by best qualitative spot resolution.

Subsequently, we used PDQuest software (BioRad, version 8.0) to compare the proteome maps of control and stressed seed. This analysis revealed a total of 339 spots differentially expressed (Student's *t*-test, $p<0.01$) among samples from control and salt-stress treatment.

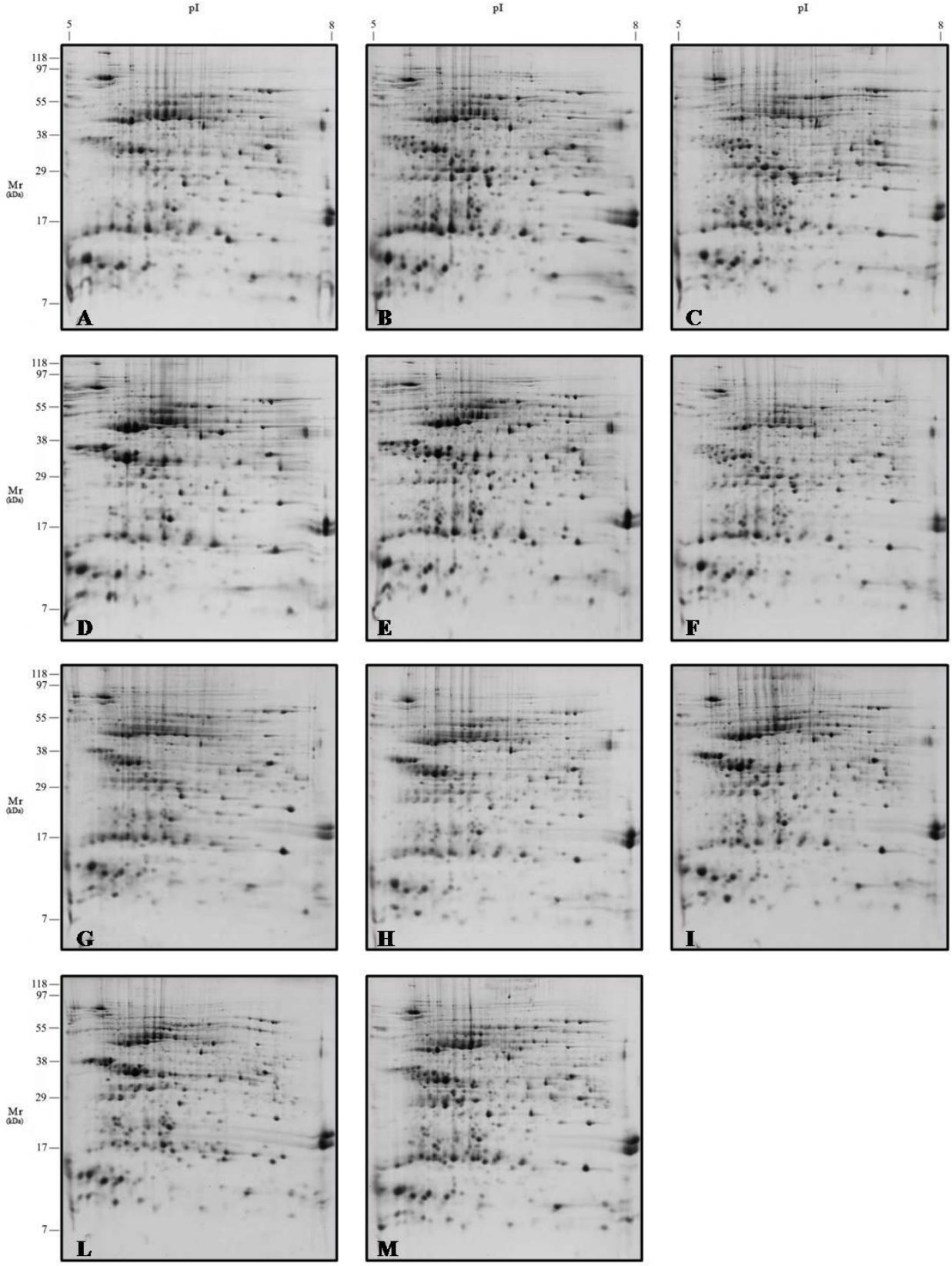

Figure 4. 2-DE maps of the *Lens culinaris* seed germinated under control conditions. Gel profiles of total proteins from: A) Capracotta 10b; B) Capracotta 14; C) Capracotta 17; D) Conca Casale 28; E) Conca Casale 30; F) Conca Casale36 (reference map); G) Castelluccio di Norcia; H) Colfiorito; I) Rascino; L) Turca rossa; M) Canadese. Gels were stained with Coomassie G250. An equal amount (700 µg) of total protein extracts was loaded in each gel.

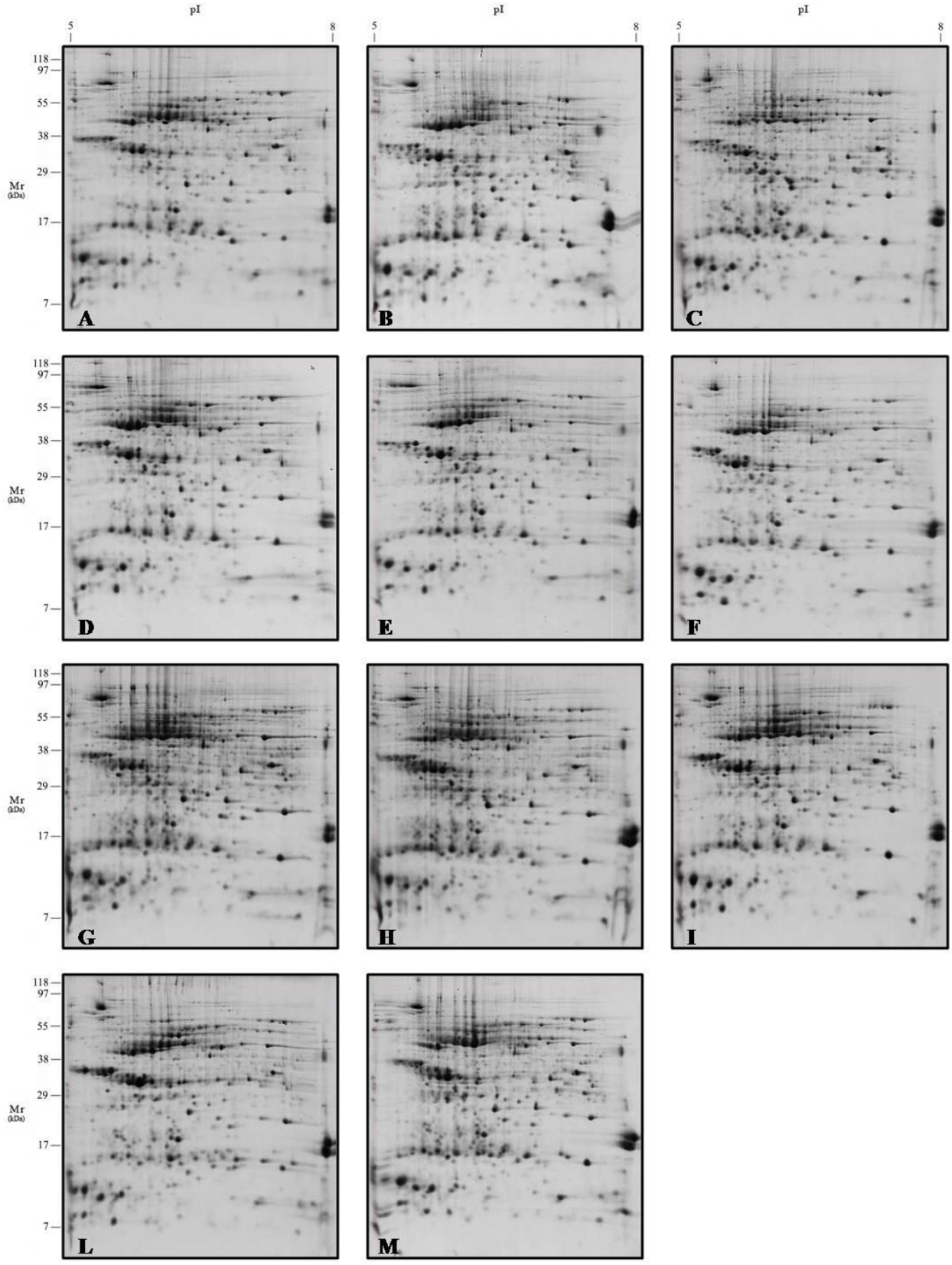

Figure 5. 2-DE maps of the *Lens culinaris* seed germinated under 200 mM NaCl stress conditions. Gel profiles of total proteins from: A) Capracotta 10b; B) Capracotta 14; C) Capracotta 17; D) Conca Casale 28; E) Conca Casale 30; F) Conca Casale36 (reference map); G) Castelluccio di Norcia; H) Colfiorito; I) Rascino; L) Turca rossa; M) Canadese. Gels were stained with Coomassie G250. An equal amount (700 µg) of total protein extracts was loaded in each gel.

2.7.3 Statistical analysis

Quantitative data of 339 protein spots resulting differentially expressed by the PDQuest analysis were subjected to analysis of variance (ANOVA, $p \leq 0.05$) and further grouped according to their similar expression profile by cluster analysis in order to detect proteins that were important to discriminate control and stressed samples.

Initially, ANOVA was performed on all 11 lentil populations analysed in this study to find protein spots that may significantly play a role in the response to salt stress in *Lens culinaris* Medik. seed. Results of the ANOVA revealed that 53 spots were involved in the response to salt stress. In detail, the cluster analysis grouped these spots in two main clusters on the base of their low or high quantity, as shown in Figure 6.

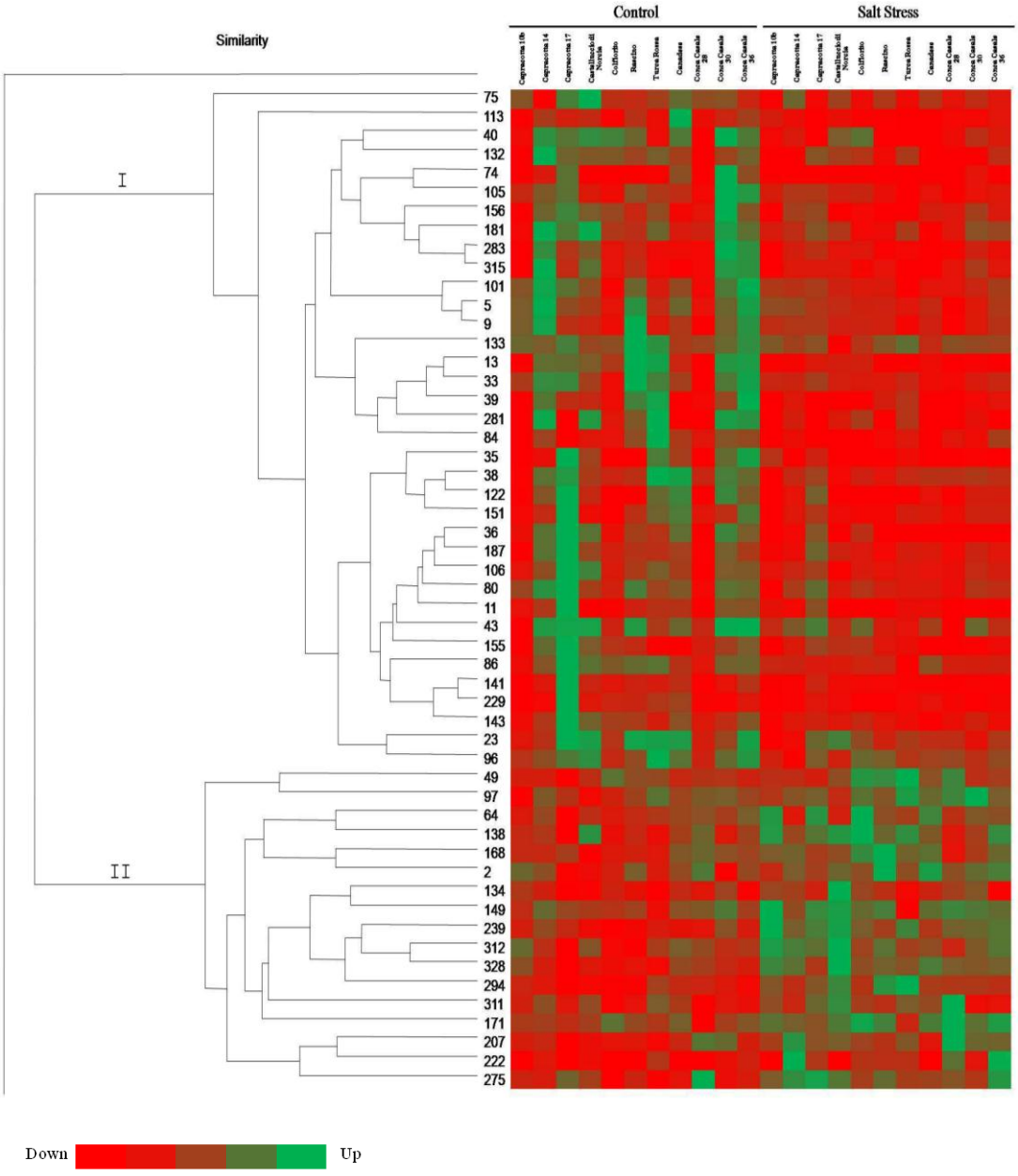

Figure 6. Hierarchical clustering of differentially expressed proteins (ANOVA, $p \leq 0.05$) between control and stressed seed of all 11 lentil populations. Arabic number on the left side refer to proteins reported in Figure 10 and Table 1.

In particular, cluster I contained 36 spots (spots 75-96) characterizing stressed samples for their quantity lower than in control, whereas cluster II grouped 17 spots (spots 49-275) more abundantly expressed in all stressed specimens (Figure 6).

Subsequently, ANOVA were carried out to distinguish protein spots which characterize the response to salt condition in Capracotta, Conca Casale lentil and other five lentil varieties (Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese).

In detail, ANOVA computed on lentil from Capracotta, revealed that 11 spots resulted significant to discriminate control from stressed samples. As shown in Figure 7, cluster analysis grouped these protein spots in two clusters: cluster I was composed of 3 spots (spots 244, 251 and 257) that were less expressed in stressed samples, whereas 8 protein spots (spots 190-325) with higher quantity in stressed samples were grouped in cluster II.

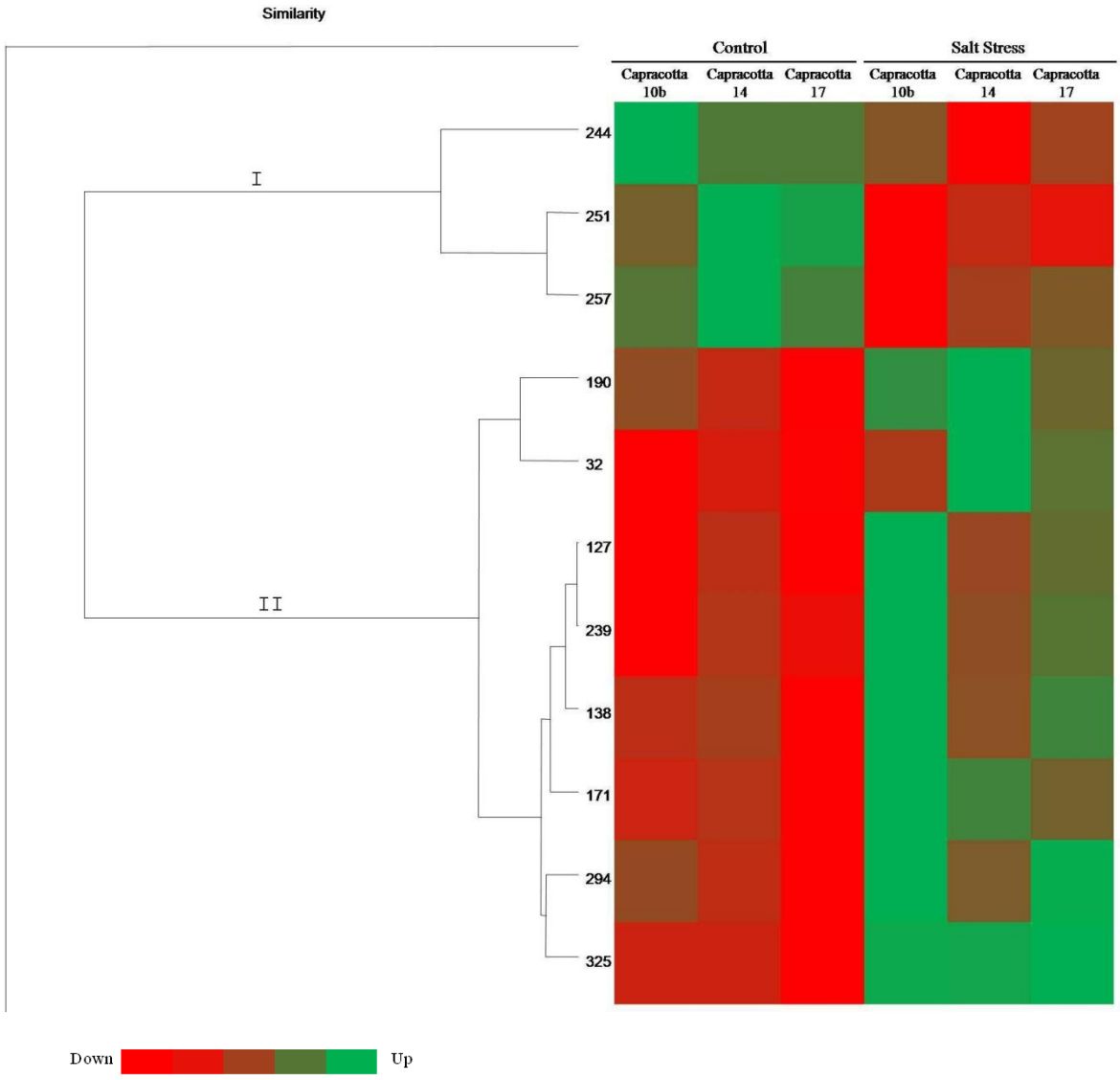

Figure 7. Hierarchical clustering of differentially expressed proteins (ANOVA, $p \leq 0.05$) between control and stressed seed of three Capracotta lentil populations. Arabic number on the left side refer to proteins reported in Figure 10 and Table 1.

For Conca Casale, ANOVA detected 14 significant spots and cluster analysis generated two main clusters (Figure 8). In particular, cluster I and cluster II were constituted by 7 low (spots 291-152) and 7 highly (spots 232-18) expressed proteins in stressed samples, respectively (Figure 8).

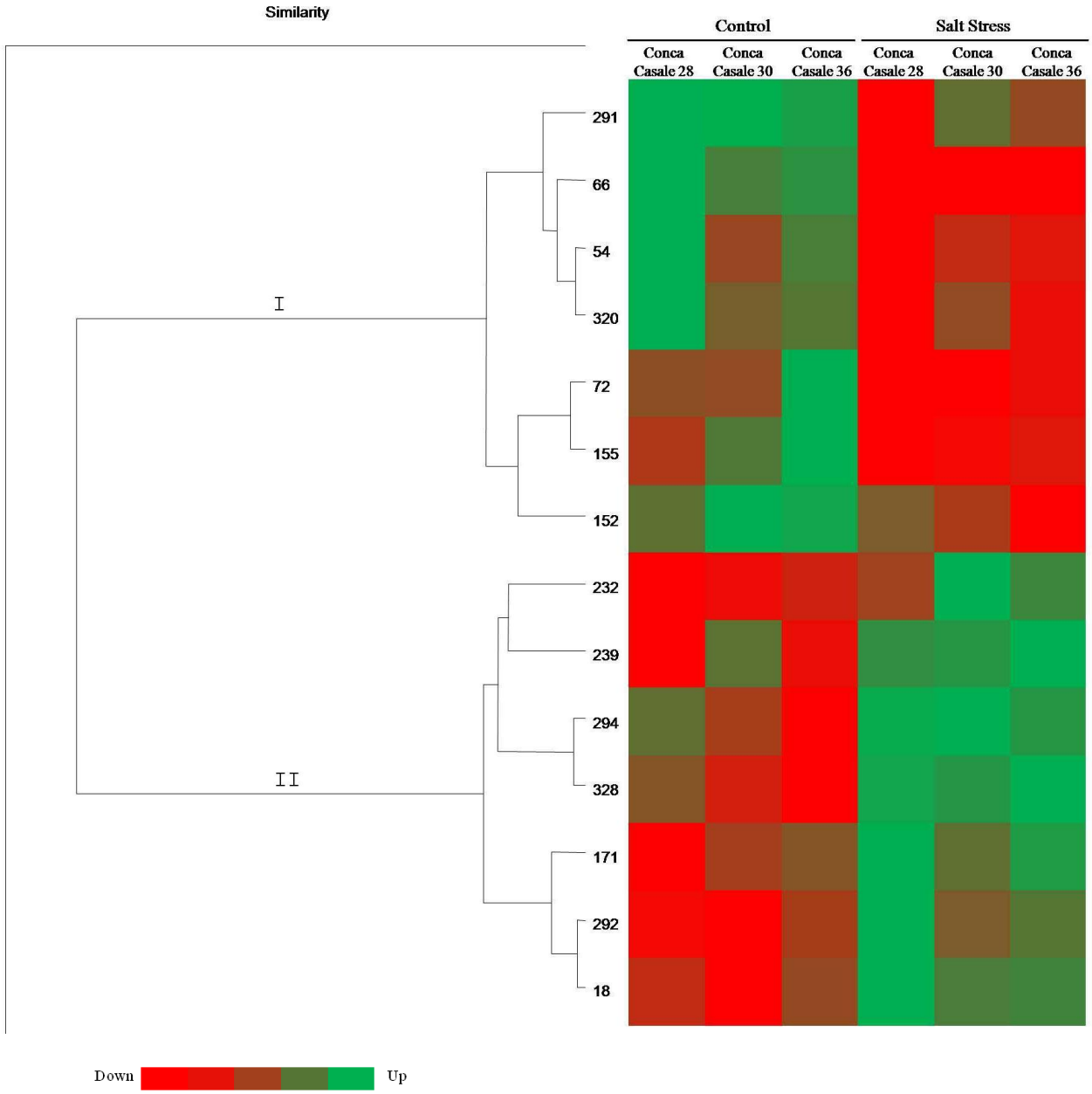

Figure 8. Hierarchical clustering of differentially expressed proteins (ANOVA, $p \leq 0.05$) between control and stressed seed of three Conca Casale lentil populations. Arabic number on the left side refer to proteins reported in Figure 10 and Table 1.

Finally, analysis of variance computed on Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese lentils revealed that 32 protein spots resulted significant for their expression to discriminate control and stressed samples (Figure 9). Cluster analysis grouped the 32 spots in two main clusters: cluster I contained 16 proteins (spots 138-328) which characterized stressed

samples for their high expression level, while cluster II included 16 spots more abundantly expressed in control samples (Figure 9).

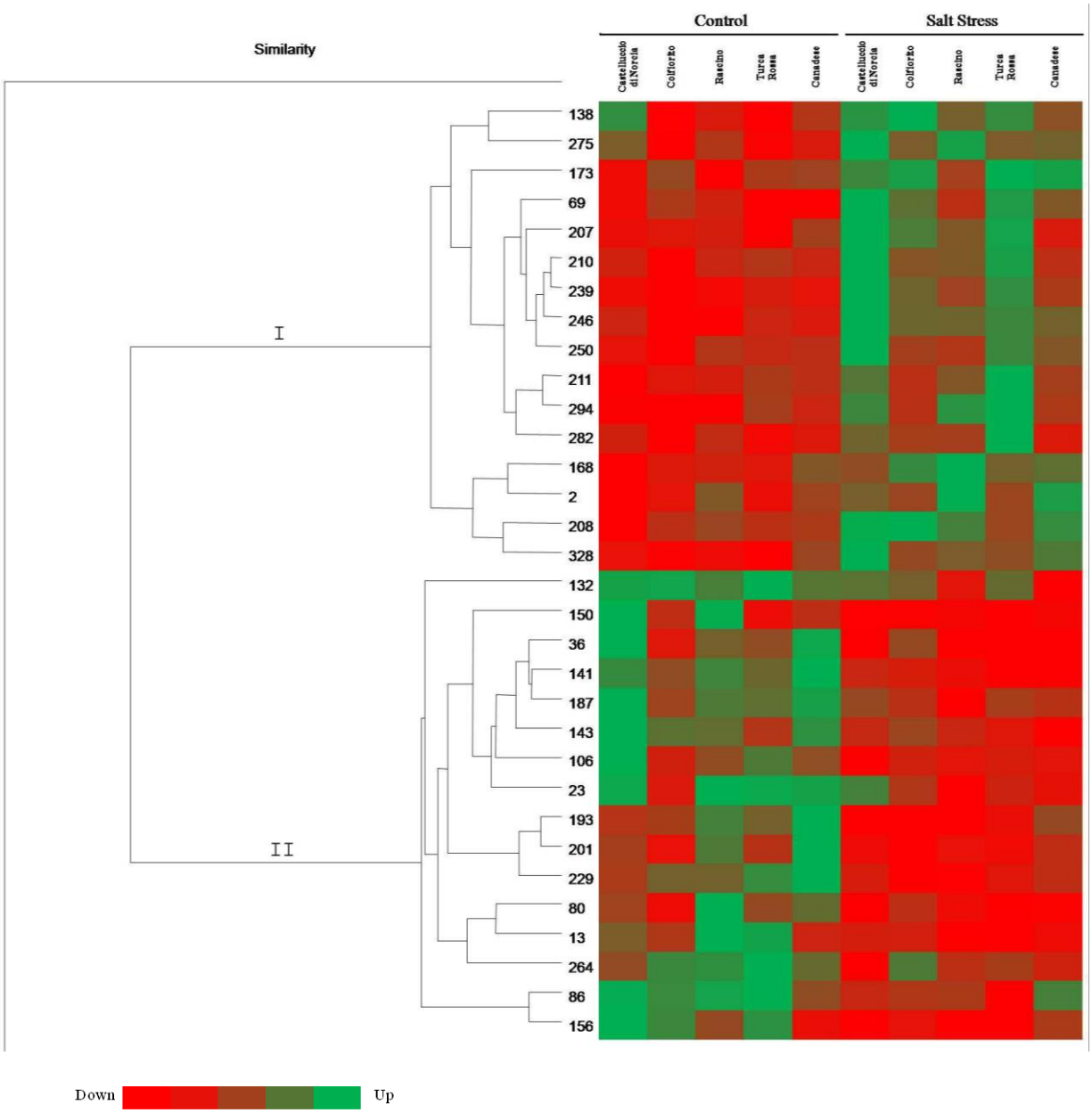

Figure 9. Hierarchical clustering of differentially expressed proteins (ANOVA, $p \leq 0.05$) between control and stressed seed of Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese lentil populations. Arabic number on the left side refer to proteins reported in Figure 10 and Table 1.

2.7.4 Identification of salt-responsive proteins

The 81 protein spots detected as discriminating by analyses of variance (Figure 10) were excised manually from gels, digested with trypsin and subjected to MALDI-TOF PMF and/or nanoLC-ESI-LIT-MS/MS to determine protein identity.

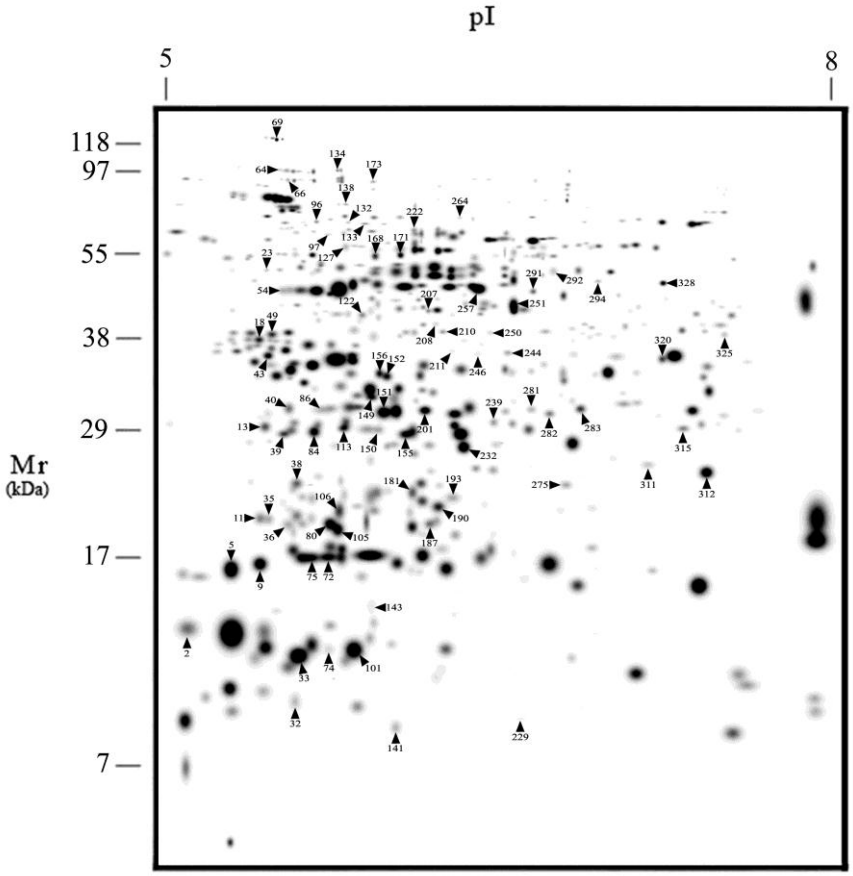

Figure 10. Two-dimensional electrophoresis reference map of *L. culinaris* germinated seeds. Protein (700 µg) were separated on pH 5-8 IPGs (first dimension), 12% SDS-PAGE (second dimension) and visualized by colloidal Coomassie blue staining. Spot numbering refers to Table 1 that shows the protein identification as ascertained by MS analysis.

By mass spectrometry analysis the 81 spots were identified as 105 proteins, which corresponded to 31 unique sequence accessions. In detail, 43 spots showed unequivocal identification, 29 spots revealed multiple identification and 9 spots (spots 64, 96, 97, 133, 134, 138, 275, 291 and 294) remained unidentified as a result of lack of genomic information for *Lens culinaris*. Furthermore, since the incomplete genome sequencing of *Lens culinaris,* 10 of homologous proteins were only described as predicted protein (spots 2, 69, 75, 122, 281), unnamed protein product (spots 132, 187) or unknown (spots 149, 168, 239). The results of protein identification are summarized in Table 1.

Table 1. Proteins identified in the 2-DE proteomic map of *L. culinaris* seeds germinated under control and salt stress conditions. Spot number, accession number, protein name, organism, theoretical and experimental pI and Mr values, sequence coverage, number of peptides matched, identification score, and function are listed. Spot numbering refers to Figure 10.

Spot number	Accession	Protein name	Organism	Theoretical pI/Mw (kDa)	Experimental pI/Mw (kDa)	Sequence coverage (%)	Peptides	Mascot Score	Functional classification
2	gi|224094719	Predicted protein	*P. trichocarpa*	9.56 / 71.44	5.09 / 12.45	9	6	215	miscellaneous
5	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.29 / 16.03	23	11	446	protein destination and storage
9	gi|137582	Vicilin	*P. sativum*	5.39 / 52.24	5.42 / 16.40	33	11	406	protein destination and storage
11	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.42 / 19.92	10	3	112	protein destination and storage
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		11	3	141	protein destination and storage
13	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.44 / 29.21	8	3	172	protein destination and storage
18	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28	5.41 / 37.65	15	4	245	protein destination and storage
23	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.45 / 51.06	7	3	111	protein destination and storage
32	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.58 / 9.13	14	4	306	protein destination and storage
	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13		15	5	234	protein destination and storage
33	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.59 / 11.09	27	18	659	protein destination and storage
35	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28	5.46 / 19.84	7	2	164	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		6	2	152	protein destination and storage
36	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.55 / 19.38	9	5	140	protein destination and storage
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		11	3	177	protein destination and storage
38	gi|2765097	P54 protein	*P. sativum*	6.05 / 54.66	5.58 / 23.08	22	10	349	disease/defence
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		15	4	204	protein destination and storage
39	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.52 / 28.41	37	12	521	protein destination and storage
40	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.55 / 30.82	14	6	244	protein destination and storage
43	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.80	5.45 / 35.93	19	7	282	protein destination and storage
49	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28	5.47 / 38.37	13	6	220	protein destination and storage
	gi|84468360	60S acidic ribosomal protein P0	*T. pratense*	5.26 / 34.33		12	3	152	protein synthesis
54	gi|137582	Vicilin	*P. sativum*	5.33 / 49.34	5.52 / 46.64	12	4,00	138	protein destination and storage
	gi|170064	Sucrose-binding protein	*G. max*	6.08 / 57.22		15	6,00	234	transporters
66	gi|310753530	Actin isoform PEAc14-1	*P. sativum*	5.23 / 41.80	5.54 / 90.83	6	2	100	cell structure
69	gi|224094719	Predicted protein	*P. trichocarpa*	9.46 / 71.44	5.49 / 119.52	7	5	152	miscellaneous
72	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.72 / 16.87	15	6	264	protein destination and storage
74	gi|170064	Sucrose-binding protein	*G. max*	6.08 / 57.22	5.73 / 11.40	6	2	104	transporters
75	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.65 / 16.84	19	8	360	protein destination and storage
	gi|222867634	Predicted protein	*P. trichocarpa*	5.70 / 18.55		10	2	109	miscellaneous
80	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28	5.73 / 19.44	21	7	330	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		11	4	164	protein destination and storage

Spot number	Accession	Protein name	Organism	Theoretical pI/Mw (kDa)	Experimental pI/Mw (kDa)	Sequence coverage (%)	Peptides	Mascot Score	Functional classification
84	gi|137582	Vicilin	*P. sativum*	5.33 / 49.34	5.66 / 28.69	35	13	547	protein destination and storage
86	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.70 / 30.71	25	11	426	protein destination and storage
	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79		15	7	295	protein destination and storage
101	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.86 / 11.37	28	17	906	protein destination and storage
105	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.77 / 18.96	13	5	228	protein destination and storage
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		12	4	261	protein destination and storage
106	gi|282925	Legumin K	*P. sativum*	5.65 / 56.29	5.78 / 20.54	19	7	332	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		12	4	177	protein destination and storage
	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13		5	2	130	protein destination and storage
113	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	5.80 / 29.08	31	11	351	protein destination and storage
122	gi|224094719	Predicted protein	*P. trichocarpa*	9.46 / 71.44	5.91 / 41.84	11	7	327	miscellaneous
	gi|11992263	Glycinin subunit G7	*G. max*	6.49 / 60.49		12	6	150	protein destination and storage
127	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.81 / 57.56	9	4	188	protein destination and storage
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		10	3	115	protein destination and storage
132	gi|257353854	Unnamed protein product	*M. x domestica*	5.44 / 60.79	5.83 / 68.10	7	3	110	miscellaneous
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		5	3	100	protein destination and storage
141	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13	6.07 / 8.17	5	5	276	protein destination and storage
143	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13	5.96 / 13.69	9	4	253	protein destination and storage
149	gi|255626763	Unknown	*G. max*	5.72 / 27.64	5.96 / 31.80	72	20	1011	miscellaneous
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		21	8	225	protein destination and storage
150	gi|137582	Vicilin	*P. sativum*	5.33 / 49.34	5.99 / 28.93	34	14	565	protein destination and storage
151	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.01 / 30.46	41	18	739	protein destination and storage
152	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	6.03 / 33.84	31	13	639	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		28	11	362	protein destination and storage
155	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.11 / 28.39	40	16	605	protein destination and storage
156	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79	5.99 / 34.12	26	10	433	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		20	7	220	protein destination and storage
168	gi|42521309	Enolase	*G. max*	5.31 / 47.72	5.97 / 54.43	25	7	299	energy
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		11	5	147	protein destination and storage
	gi|217073039	Unknown	*M. truncatula*	5.68 / 36.82		10	3	146	miscellaneous
171	gi|42521309	Enolase	*G. max*	5.31 / 47.72	6.09 / 54.63	43	13	730	energy
173	gi|118573101	Putative poly [ADP-ribose] polymerase 3	*M. truncatula*	5.20 / 89.99	5.96 / 89.86	11	5	210	disease/defence
181	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.14 / 22.21	21	6	237	protein destination and storage
187	gi|297735192	Unnamed protein product	*V. vinifera*	8.51 / 53.25	6.22 / 19.43	11	5	296	miscellaneous
190	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28	6.26 / 20.90	21	7	397	protein destination and storage
	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13		14	5	257	protein destination and storage

Spot number	Accession	Protein name	Organism	Theoretical pI/Mw (kDa)	Experimental pI/Mw (kDa)	Sequence coverage (%)	Peptides	Mascot Score	Functional classification
193	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.32 / 21.73	27	9	195	protein destination and storage
	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13		18	7	308	protein destination and storage
	gi|282925	Legumin K	*P. sativum*	5.65 / 56.28		18	5	237	protein destination and storage
201	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.20 / 30.64	35	16	532	protein destination and storage
207	gi|9230771	Cytosolic phosphoglycerate kinase	*P. sativum*	5.73 / 42.29	6.21 / 42.61	13	3	120	energy
208	gi|2765097	P54 protein	*P. sativum*	6.05 / 54.66	6.24 / 40.19	17	5	200	disease/defence
210	gi|42521311	Cytosolic malate dehydrogenase	*G. max*	6.32 / 35.53	6.28 / 38.82	11	4	308	energy
211	gi|137582	Vicilin	*P. sativum*	5.33 / 49.34	6.31 / 36.41	15	5	172	protein destination and storage
222	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13	6.15 / 64.71	21	8	317	protein destination and storage
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		8	3	100	protein destination and storage
229	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13	6.60 / 8.48	12	7	269	protein destination and storage
232	gi|113570	Albumin-2	*P. sativum*	5.16 / 26.24	6.36 / 26.89	71	19	951	protein destination and storage
	gi|77540216	Triosephosphate isomerase	*G. max*	5.87 / 27.20		24	4	229	energy
239	gi|255628279	Unknown	*G. max*	6.42 / 27.05	6.49 / 29.59	52	10	449	miscellaneous
	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23		21	6	214	protein destination and storage
244	gi|6525021	Isoflavone reductase-like NAD(P)H-dependent oxidoreductase	*M. sativa*	6.46 / 33.97	6.55 / 36.23	19	5	138	disease/defense
246	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.42 / 36.13	7	3	83	protein destination and storage
250	gi|42521311	Cytosolic malate dehydrogenase	*G. max*	6.32 / 35.53	6.18 / 38.63	11	3	213	energy
251	gi|11992263	Glycinin subunit G7	*G. max*	6.49 / 60.49	6.58 / 43.58	11	5	114	protein destination and storage
257	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	6.43 / 46.99	37	16	672	protein destination and storage
264	gi|255579273	Succinate dehydrogenase, putative	*R. communis*	6.18 / 68.51	6.35 / 70.14	4	2	137	energy
281	gi|224094719	Predicted protein	*P. trichocarpa*	9.46 / 71.44	6.65 / 30.73	13	7	247	miscellaneous
	gi|11992263	Glycinin subunit G7	*G. max*	6.49 / 60.49		10	5	116	protein destination and storage
282	gi|2270994	Ca+2-binding EF hand protein	*G. max*	5.98 / 26.98	6.73 / 30.35	11	3	122	disease/defense
283	gi|11992263	Glycinin subunit G7	*G. max*	6.49 / 60.49	6.86 / 30.77	13	6	155	protein destination and storage
	gi|4218520	LegA class precursor	*P. sativum*	6.16 / 58.79		7	3	131	protein destination and storage
292	gi|137582	Vicilin	*P. sativum*	5.33/49.34	6.75 / 50.88	39	14	617	protein destination and storage
311	gi|75323225	1-cys peroxiredoxin	*M. truncatula*	6.08 / 24.41	7.16 / 24.96	53	13	273	disease/defence
312	gi|75323225	1-cys peroxiredoxin	*M. truncatula*	6.08 / 24.41	7.43 / 24.16	22	4	170	disease/defence
	gi|310753530	Actin isoform PEAc14-1	*P. sativum*	5.23 / 41.80		8	2	103	cell structure
	gi|159895414	Actin 1	*Z. jujuba*	4.65 / 17.56		18	2	287	cell structure
315	gi|126170	Legumin J	*P. sativum*	5.70 / 56.90	7.32 / 29.06	7	4	89	protein destination and storage
320	gi|7688242	Convicilin	*L. culinaris*	5.56 / 60.13	7.23 / 35.59	18	11	626	protein destination and storage
325	gi|1345501	Glyceraldehyde-3-phosphate dehydrogenase	*A. majus*	8.30 / 36.69	7.51 / 38.32	28	6	331	energy
	gi|1168410	Fructose-bisphosphate aldolase	*P. sativum*	6.77 / 38.49		7	2	63	energy
328	gi|137582	Vicilin	*P. sativum*	5.39 / 52.23	7.23 / 48.21	8	2	124	protein destination and storage
	gi|11363146	IAA-protein conjugate	*P. vulgaris*	6.20 / 35.52		4	1	110	miscellaneous

The 105 identified proteins were divided into 7 different functional categories according to Bevan's classification (1998), as shown in Figure 11 and Table 1. The most represented class was that of protein destination and storage (68%), followed by proteins involved in energy (9%), disease/defence (7%), cell structure (3%), transporters (2%), protein synthesis (1%) and miscellaneous (10%) (Figure 11). The class of "miscellaneous" proteins included proteins identified like predicted protein, unnamed protein product, unknown, and proteins that couldn't be grouped into the classes above mentioned.

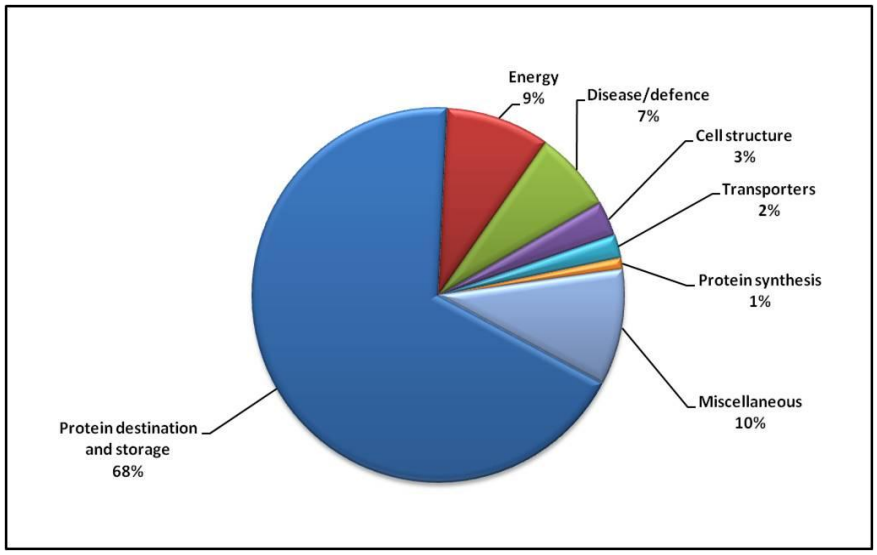

Figure 11. Functional classification of proteins identified in *Lens culinaris* seeds germinated under control and salt stress conditions according to nomenclature by Bevan et al. (1998).

2.8 Discussion

2.8.1 Physiological responses induced by salt stress

In the present study, we evaluated the effect of salinity on seed germination of two lentil landraces from Molise and five commercial varieties. Lentil seeds were germinated in presence of different NaCl solutions; in particular, 100 mM and 200 mM NaCl solutions were used since many researchers reported that lower concentrations of salt didn't affect significantly germination (El-Monem & Sharaf, 2008; Sidari et al., 2008).

Seed germination is one of the most important phases in the life cycle of plant and is highly responsive to existing environment (Kuriakose & Prasad, 2007).

Salinity affects seed germination through osmotic effects (Bliss et al., 1986; Kafi & Goldani, 2001; Jamil & Rha, 2004), ion toxicity (Jones, 1986; Hampson & Simpson, 1990) or a combination of the two (Huang & Redmann, 1995).

It is assumed that germination rate and the final seed germination decrease with the decrease of the water movement into the seeds during imbibitions (Hadas, 1977).

Younis et al. (1991) reported that low moisture content under salt stress caused cessation of metabolism or inhibition of certain steps in metabolic sequences of germination. Evenly, during germination, salt stress increased the intake of toxic ions which may alter certain enzymatic (Lin & Kao, 1995; Almasouri et al., 1999) or hormonal activities of seeds (Smith & Comb, 1991). Moreover, several reports suggest that hyper-saline environments cause delayed germination (Prado et al., 1995) by reducing hydrolytic enzyme activities and retarding the mobilization rate of metabolites (Ashraf et al., 2002).

High intracellular concentrations of both Na and Cl can inhibit the metabolism of dividing and expanding cells (Neumann, 1997), retarding germination and even leading to seed death.

The results obtained from the germination tests showed that the two different salt concentrations have a diverse effect on germination rate and percentage. Futhermore, differences have been observed within and between all the populations analysed. In general, 100 mM NaCl solution affects mainly the germination speed (T_{50}) with a consequent delay of germination of all the 11 populations. In fact, the final germination percentage was little reduced in the majority of the samples. However, in Capracotta 10b, Turca Rossa and Canadese, 100 mM NaCl seems to promote even slightly the germination as indicate by the increase of their germination percentage. Under 200 mM NaCl conditions, both the final germination percentage and germination rate are strongly affected in all studied populations. The effects reported under low salt condition (100 mM NaCl) can be attributed to a decrease water absorption, that affect the synthesis of hydrolitic enzymes limiting the hydrolysis of food reserves from storage tissues as well as to impaired translocation of food reserves from storage tissue to developing embryo axis (Dubey, 1985; de Lacerda et al., 2003). However, it can be also hypothesized that the presence of NaCl at low concentrations, which is penetrating ions, could have contributed to a decrease in the internal osmotic potential of germinating structures, as suggested by Dodd & Donovan (1999) and Almansouri et al. (2001) leading to water uptake and initiation of germination processes (Sidari et al., 2008).

The significant increase of T_{50} and decrease of germination percentage observed under conditions of high salt concentration (200 mM NaCl), may be attributed to the combination of "osmotic effect" and ion toxicity.

These results are in line with those obtained in other studies which report a decrease in germination percentage and a delay in germination of different plant species with increasing NaCl solution (Bayuelo-Jimenez et al., 2002; Maghsoudi & Maghsoudi, 2008; Bybordi & Tabatabaei, 2009; Hakim et al., 2010; Dkhil & Denden, 2010; Asgharipour & Rafiei, 2011; Abazarian et al., 2011).

Moreover, our results also show differences in germination percentage and rate within populations of the same landrace and between landraces and commercial varieties. Although the three different populations of each local landrace have rather heterogeneous responses to the salt treatments it can be suggested that they are better equipped than the three Italian commercial varieties to respond to salinity stress. On the other hand, the two commercial varieties Canadese and Turca Rossa perform very similarly to the autochthonous landraces. The variation in stress sensitivity has been attributed to different causes. In a recent work it has been proposed that the variation in stress sensitivity of different lentil genotypes may be linked to their ability of osmoregulating. In fact, it has been observed that under salt stress, which causes a strong decrease in water content, the hydrolytic enzyme activities are strongly affected and in turn the hydrolysis of food reserves (Sidari et al., 2008). Based on these evidences it can be suggested that different populations and ecotypes are characterised by a diverse capacity to osmoregulate under salt stress conditions.

2.8.2 Alteration in seed proteome under salt stress

Improvements in proteomic technology regarding protein separation and detection as well as mass spectrometry-based protein identification, had an increasing impact on the study of plant responses to salinity stress (Parker et al., 2006; Qureshi et al., 2007; Caruso et al., 2008). New insights have been obtained into salinity stress response by comparative proteome studies of salt-stressed roots from *Pisum sativum* (Kav et al., 2004), *Oryza sativa* (Yan et al., 2005), Arabidopsis (Jiang et al., 2007), barley (Witzel et al., 2009) and *Glycine max* (Sobhanian et al., 2010a). Further studies were carried out to analyze changes in protein expression caused by salt stress in *Glycine max* leaves and hypocotyls (Aghaei et al., 2009; Sobhanian et al., 2010a), in wheat leaves (Gao et al., 2011) and in seedling from tomato and soybean (Chen et al., 2009; Ma et al., 2012).

Very few studies are reported in the literature on the use of proteomic approach to investigate the response of seed to salinity conditions; in particular, as far as we know, only the recent paper published by Xu et al., (2011) reports the proteomic analysis of seed germination under salt stress in soybean where 650 spots were reproducibly resolved using pH 3-10 pH 4-7 ranges, of which 18 proteins spots showed changes in abundance as a result of salt stress, but only 9 proteins were successfully identified.

Lentil is a strategic crop from an agronomic and food point of view. In fact, it is an important component of the cropping system in the Mediterranean areas and precious proteins sources for human and animals. This species is classified as salt sensitive (Ashraf & Waheed, 1990). The

identification of salt tolerant lentil genotype is very important for the cultivation on saline soils or with saline waters, conditions very frequent in the Mediterranean areas.

In a previous work carried out within this PhD program and published in the paper Scippa et al. (2010), the map of the proteome of *Lens culinaris* mature seed was produced, whereas the modifications in the protein patterns of lentil embryo axes germinating under salt stress was evaluated by Dell'Aquila (2004). Any information wasn't available about salt response in germinating lentil seed, therefore our work originally reports a detailed proteomic analysis to identified salt stress responsive proteins in seed germination in two lentil landraces from Molise (Capracotta and Conca Casale) and five commercial varieties.

We identified 105 spots, which corresponded to 31 total protein accessions (43 unequivocal and 29 multiple identification accessions, Table 1). Several identified proteins exhibited remarkable differences between experimental and theoretical molecular weights (Table 1). In addition, the theoretical pIs of 7 proteins (spots 2, 69, 122, 187, 281, 312 and 325) were beyond the pH range of the strips we used (Table 1). The phenomena have also been reported for the protein profiles in citrus flesh (Muccilli et al., 2009), peach fruit (Zhang et al., 2011) and soybean seedling (Ma et al., 2012), and may be due to numerous factors, such as expression in different organisms, different gene expression products, post-translational modifications or degradation of the same gene expression. The lack of recognition of several spots (9 in number) and the identification of similar proteins from different plant species could be due to the paucity of information about *L. culinaris* genome in agreement with our previous work (Scippa et al., 2010).

Proteins identified in this study fell into 7 different functional classes (Figure 11) where protein destination and storage represented the most abundant, followed in order by energy, disease/defence, cell structure, transporters, protein synthesis and miscellaneous.

Comparing the proteome profiles in control and salt treated seeds of all the 11 studied populations revealed that 200 mM NaCl treatment induced considerable changes in the pattern of proteins; in fact, 339 protein spots resulted differentially expressed. In order to detect proteins mainly involved in response to salt stress in germinating lentil seed, the ANOVA statistical analysis followed by hierarchical clustering was carried out on the quantitative data of differential spots. Results showed that 53 proteins had significant changes in abundance in response to 200 mM NaCl stress. In particular, 36 spots were down-regulated by salt stress in all populations, whereas 17 protein spots were more expressed in stressed seed (Figure 6). Among these protein spots, 24 spots had unequivocal identification, 21 showed multiple identification and 8 were unidentified.

Notably, salt stress mostly affected the storage protein content. In particular, the majority of proteins corresponding to 7S and 11S globulins significantly decreased in seeds under salt conditions, although in few cases an increase was observed.

Seed storage proteins are functionally involved in providing the developing seedling with nitrogen, carbon and sulphur during seed germination (Shewry et al., 1995). Moreover, several studies reported that storage proteins can have a role in protecting seeds from environmental stresses (Sales et al., 2000; Agizzio et al., 2003). In fact, it was demonstrated that vicilins protect seeds from pathogens during germination (Macedo et al., 1993; Chung et al., 1997) by delaying larval development and growth of spores of phytopathogenic fungi (Gomes et al., 1997; Yunes et al., 1998; Sales et al., 2001).

The decreases of storage proteins occurring in all the lentil populations analysed under salt stress conditions may be attributed to two different response mechanisms. As storage proteins are the source of energy their decrease may be linked to the higher energy requirement to face stress. However, their degradation might be require for an osmoregulatory mechanism.

Among the differentially expressed proteins in response to salt stress, two were involved in disease/defence reactions, namely, P54 protein (spot 38) and 1-cys peroxiredoxin (spots 311 and 312). The former was more abundant in control seeds, whereas the latter was more expressed in stressed samples.

P54 protein has been proposed to be involved in the protection of chromatin structure against desiccation during seed maturation (Chiatante et al., 1995; Castillo et al., 2000; Scippa et al., 2011). It has been reported that psp54 gene is differentially induced by abscisic acid as well as by water stress conditions (Castillo et al., 2005); however here we show that it is significantly downregulated in seeds subjected to salt stress treatment. Although water and saline stress may share common response pathways, there are also specific factors that are differently modulated by these two conditions. It can be proposed that under salt stress chromatin is protected by other factors rather than P54, or that its decrease is due to the toxic effect of the salt.

A study carried out on Arabidopsis mutant lines showed that the seeds characterized by an overexpression of 1-cys peroxiredoxin were less inclined to germinate than wild-type seeds in the presence of NaCl. These results suggested that 1-cys peroxiredoxin can be involved in sensing harsh environmental surroundings and play a part in the regulation of germination timing under unfavorable conditions (Haslekås et al., 2003). This role may also be played in lentil seeds subjected to 200 mM salt treatment when a reduction of final germination has been observed.

A general up-regulation of proteins related to energy was found; in particular, enolase (spots 168 and 171) and cytosolic phosphoglycerate kinase (spot 207) were more abundant in stressed than in control seeds.

Enolase is an essential and ubiquitous enzyme that catalyzes the Mg^{2+}-dependent dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP), which is converted by pyruvate kinase into pyruvate with the concomitant generation of ATP in the subsequent final step of glycolysis (Hannaert et al., 2000). The up-regulation of enolase has been previously reported in rice, cucumber and tomato roots in response to salt stress (Yan et al., 2005; Du et al., 2010; Manaa et al., 2011). The existence of multiple enolase isoforms in plants has been reported (Van der Straeten et al., 1991) and it is possible that the two enolases identified in this study represent different isoforms.

Phosphoglycerate kinase utilizes ATP to phosphorylate 3-phosphoglycerate to form 1,3 bisphosphoglycerate. This reaction represents the first reaction in the reduction step of the Calvin cycle (Caruso et al., 2008). Phosphoglycerate kinase levels were enhanced by salt stress in rice leaf laminas (Parker et al., 2006) and in root of *Leymus chinensis* (Jin et al., 2008), suggesting that this enzyme is an early responsive protein to salt stress. Conversely, it was observed a decrease in expression of this enzyme in leaf of *Triticum durum* (Caruso et al., 2008) and in root of tomato (Manaa et al., 2011).

The accumulation of these proteins may be related to an increase of glycolytic activity, probably due to a need for the extra energy supply necessary for germination of lentil seeds under salt stress.

Two other up-regulated proteins in lentil seeds under salt stress were found to be involved in cell structure: actin (spot 312 with multiple identification) and actin isoform PEAc14-1 (spot 312 with multiple identification). Actin is a component of the cytoskeletal system that allows movement of cells and cellular processes, and it may play an important role in salt stress tolerance mechanims (Wang et al., 2011; Pollard & Cooper, 2009). In fact, salt stress causes ion imbalance and hyperosmotic stress in plants. Thus actin might play an important role in cytoskeleton reorganization.

Ribosomal proteins are named after the subunit of the ribosome in which they are found and for the size of their molecules. In the yeast *Saccharomyces cerevisiae*, the ribosomal protein consists of a large 60S subunit and a small 40S subunit, and it performs protein synthesis (Venema & Tollervey, 1999). Spot 49 was identified as 60S acidic ribosomal protein P0 and was shown to accumulate under salt stress. According to Zörb et al. (2004), the synthesis of ribosomal proteins represents the functional restructuring of the protein synthesis apparatus in maize plants under abiotic stress. Therefore, we can hypothesized that 60S acidic ribosomal protein P0 played an active role in maintaining protein metabolism in lentil seeds when exposed to salinity.

Spot 328 was identified as IAA-protein conjugate, the most abundant protein modified by the phytohormone indole-3-acetic acid (IAA) (Walz et al., 2002), and its expression level increased under salt stress. The expression of IAP1 is correlated to the developmental period of rapid growth during seed development, whereas its rapid degradation occurs during germination (Walz et al., 2002). Very little is known about the function of protein conjugates with IAA, but evidence has been presented that auxin conjugates could be involved in abiotic stress tolerance (Walz et al., 2008). Indeed the overexpression of this auxin conjugate in Arabidopsis also rendered these transgenic lines more salt tolerant (Ludwig-Müller, 2011). Since IAA-protein conjugates function as auxin storage, its transport or inactivation may have the important role in safeguarding the supply of critically important auxin to the seed.

A down-expressed protein under salt stress was identified as sucrose-binding protein (spot 74). This protein is involved in sucrose transport and it is deposited in the cells of developing cotyledons as well as mesophyll cells of young sink leaves and the companion cells of mature phloem (Grimes et al., 1992). Moreover, sucrose binding proteins (SBPs) have been shown to localize in the lumen of protein storage vacuoles of various seeds (Wang et al., 2009). Several studies have demonstrated that sucrose-binding proteins bear sequence and structural similarity to the vicilin-like seed storage proteins (Braun et al., 1996; Overvoorde et al., 1997; Heim et al., 2001; Contim et al., 2003). It has been shown that, similar to seed storage proteins, the SBPs of soybean, faba bean, pea and mung bean all gradually accumulate during seed development and are subsequently mobilized upon seed germination (Heim et al., 2001; Elmer et al., 2003; Wang et al., 2009). Although these observations could implicate functional analogy to seed storage proteins, compelling evidence in the literature indicates that members of the SBP family may perform distinct functions (Braun et al., 1996; Overvoorde et al., 1997). We may hypothesize that high concentrations of both Na^+ and Cl^- absorbed by seeds can inhibit the sucrose transport into seed cells, retarding and reducing germination.

2.8.3 Response of Capracotta lentil landraces to salt stress

ANOVA detected 11 spots that characterize response of Capracotta lentil landrace to salt stress (Figure 7). Among these protein spots, 6 spots had unequivocal identification, 5 showed multiple identification and 2 remained unidentified.

The level of several storage proteins was altered by 200mM salt stress treatment in Capracotta landrace. The probable function that storage proteins play in response to salt stress were described in previous session 2.8.2.

However, proteins associated with metabolism and energy production were induced by salt stress in Capracotta seed and their expression might reflect a major metabolic request to

counteract salinity. In detail, spot 171 was identified as enolase, while spot 325 (spot with multiple identification) was identified as glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase. All these proteins are enzymes that catalyze different steps of glycolysis.

Increased expression of enolase has been reported under hypoxic conditions that limit oxidative phosphorylation of ADP and favor ATP regeneration via glycolysis and the ethanol fermentative pathway (Zhao et al., 2004). Jeong et al. (2001) reported that gene transfer of glyceraldehyde-3 phosphate dehydrogenase improved salt resistance in transgenic potato (*Solanum tuberosum* L.) plants. FBP aldolase, a widely distributed enzyme, is the other key constituent of the glycolytic pathway, induced by salt stress. It plays an important role in carbohydrate metabolism and in the production of triose phosphates and derivatives important in signal transduction pathways (Schaeffer et al., 1997). According to Konishi et al. (2005), FBP aldolase is involved in gibberellin A-stimulated rice (*Oryza sativa* L.) root growth through activation of the glycolytic pathway.

Enhanced glycolysis and increased expression of related enzymes under abiotic stresses have been reported (Suzuki et al., 2005). In the present study, up-regulation of enolase, glyceraldehyde-3-phosphate dehydrogenase and fructose-bisphosphate aldolase might be related with the tolerance of Capracotta lentil seeds to NaCl stress.

A spot 244 identified as isoflavone reductase-like NAD(P)H-dependent oxidoreductase, was down regulated by salt treatment.

Isoflavone reductases (IFR) are the founding members of the PIP family of NADPH-dependent reductases that are involved in the biosynthesis of plant defense metabolites such as isoflavonoids (Oommen et al., 1994; Dixon, 2001). Several other homologous reductases that catalyze undetermined enzymatic reactions are also included within this family (Shoji et al., 2002). The members of this latter group are collectively called IRLs (for isoflavone reductase-like proteins) and knowledge of their function in plants is yet very limited (Brandalise et al., 2009). Insights from the published literature, however, suggest the involvement of these reductase-like proteins in plant response to biotic and abiotic stress (Blount et al., 1993; Petrucco et al., 1996; Lers et al., 1998; Dixon, 2001; Salekdeh et al., 2002b; Kim et al., 2003a, b). However, recently Sobhanian et al. (2010a, 2011) observed that expression of isoflavone reductase was negatively affected by salt stress in soybean seedlings and suggested that its down regulation indicated that flavonoid compounds do not have a main role in tolerance to salt stress in soybean seedlings. We can give the same explanation for down-regulation of isoflavone reductase-like NAD(P)H-dependent oxidoreductase in Capracotta lentil seeds under salt stress.

2.8.4 Response of Conca Casale lentil landraces to salt stress

Results obtained by ANOVA for Conca Casale lentil showed that 14 spots were discriminant between control and stressed seeds (Figure 8). In particular, 9 spots had unequivocal identification, 5 showed multiple identification and 2 were not identified. Of these proteins, 11 were classified as storage proteins (vicilin, convicilin, legA class precursor, albumin-2, legumin K) and their expression was altered in response to salt stress.

As for Capracotta proteins involved in energy and metabolism namely enolase (spot171) and a triosephosphate isomerase (spot 232 with multiple identification) were up-regulated under salt condition in Conca Casale ecotype. As previously discussed for enolase also triosephosphate isomerase is an important enzyme for glycolysis. In fact it catalyses the essential isomerisation reaction between dihydroxyacetone phosphate and D-glyceraldehyde-3-P in the glycolysis pathway (Gao et al., 2011). Several enzymes of this ATP-generating pathway, including triosephosphate isomerase, were shown to be induced upon saline and water stress in cultured cells of rice (Umeda et al., 1994). This coordinated induction is thought to be essential for activation of the entire energy-producing pathway to maintain homeostasis in stressed cells (Riccardi et al., 1998). In addition, up-regulation of triosephosphate isomerase under salt stress was described only for rice roots (Chitteti & Peng, 2007) and *A. lagopoides* shoots (Sobhanian et al., 2010b).

Furthermore, an IAA-protein conjugate (spot 328 with multiple identification), was induced by salt. Function of IAA-protein conjugate was previously illustrated, and as reported in our previous work (Ialicicco et al., 2012, in preparation) this protein has been proposed to be a specific marker of Conca Casale landrace.

A sucrose-binding protein (spot 54 with multiple identification) previously described was downregulated under salt stress condition, together with spot 66 corresponding to an actin isoform PEAc14-1 (spot 312) which was found to be upregulated. The different expression of two actin isoforms, may be related to their specific role in cytoskeleton plasticity in response to salt stress condition.

2.8.5 Response of commercial varieties of lentil to salt stress

ANOVA results detected 32 spots characterizing the response of commercial varieties of *Lens culinaris* to salt condition (Figure 9). Among these protein spots, 22 spots showed unequivocal identification, 5 had multiple identification, while 3 remained unidentified.

All proteins with lower expression level under 200 mM NaCl stress were storage proteins (vicilin, legA class precursor, legumin K, convicilin) with the exception of spot 264 which was identified as succinate dehydrogenase.

Succinate dehydrogenase is a membrane-bound component of the respiratory chain of aerobic organisms (Ackrell et al., 1992). It couples the reduction of ubiquinone to the oxidation of succinate and is, as such, a Krebs cycle as well as a respiratory chain enzyme. There is evidence that the respiration rate of mitochondria isolated from salt-stressed seedlings of durum wheat (*Triticum durum*) or barley (*Hordeum vulgare*) can be significantly decreased (Jolivet et al., 1990; Flagella et al., 2006; Trono et al., 2004). This suggests that the ionic component of salinity stress exerts a toxic effect on the mitochondrial electron transport chain, impairing the activity of succinate dehydrogenase in seeds of commercial lentil varieties exposed to high NaCl concentration.

Among up-regulated proteins, we identified five vicilin isoforms (spots 168, 211, 239, 246, 328), a cytosolic phosphoglycerate kinase (spot 207), two cytosolic malate dehydrogenase (spots 210 and 250), an enolase (spot 168), a P54 protein (spot 208), a putative poly [ADP-ribose] polymerase 3 (spot 173), a Ca^{+2}-binding EF hand protein (spot 282) and an IAA-protein conjugate (spot 328).

The function and the putative role that cytosolic phosphoglycerate kinase, enolase, P54 protein and IAA-protein conjugate play in response to salt stress described in previous session 2.8.2.

Malate dehydrogenase catalyzes the interconversion of oxaloacetic acid and malate and exists in various isoforms (Gietl, 1992). The isoforms localized in subcellular organelles like peroxisomes, mitochondria and cytosol are NAD-dependent whereas the chloroplastic one is NADP-dependent (Gietl, 1992). Differential expression of malate dehydrogenase isoforms and changes in its activity have been reported in many plant species under abiotic stresses (Ivanishchev, 1997; Kingstonsmith et al., 1997). Weimberg (1967) found that an isoenzyme of malate dehydrogenase isolated from pea seeds, was stimulated by NaCl if the concentration of the salt did not exceed 20 mM; higher concentration of salts were inhibiting.

We suggest that overexpression of this enzyme might promote tolerance of commercial varieties lentil seeds to salt condition.

Poly(ADP-ribose) polymerase (PARP) is a highly conserved enzyme involved in multiple aspects of plant cell physiology. For example, PARP is thought to be intimately involved in the early signaling events that trigger the DNA damage response (Semighini et al., 2006). PARP is localized in the nucleus and is activated by DNA strand breaks (Babiychuk et al., 1998; Doucet-Chabeaud et al., 2001) caused by, for instance, reactive oxygen species (De Block et al., 2005). Experiments in Arabidopsis have shown that DNA strand breaks, caused by ionizing radiation or oxidative stress, induce rapid and massive accumulation of PARP (Doucet-Chabeaud et al., 2001).

High expression level of PARP in NaCl-stressed lentil seeds might be linked to production of reactive oxygen species induced by salt stress and might increase adaptation capacity of lentil seeds to salinity.

Tolerance or susceptibility to salt stress in plants is a coordinated action of various genes including those encoding calcium-binding proteins (Mahajan et al., 2008). Three out of the four classes of Ca^{2+}-binding proteins in plants contain Ca^{2+}-binding EF-hand motif(s). This motif is a conserved helix-loop-helix structure that can bind a single Ca^{2+} ion (Day et al., 2002). Jang et al. (1998) showed that a Ca^{2+}-binding protein encoded in *Arabidopsis thaliana* was induced by NaCl stress. We propose that up-regulation of Ca^{+2}-binding EF hand protein may be related to salinity stress tolerance in commercial lentil varieties.

2.9 Conclusion

In this work the response of lentil (*Lens culinaris*) seeds of two autochthonous landraces of Molise (Capracotta and Conca Casale) and five commercial varieties (Castelluccio di Norcia, Colfiorito, Rascino, Turca Rossa and Canadese) to salt stress during germination was investigated at both physiological and proteomic level. Lentil seeds were exposed to 100 mM and 200 mM NaCl until radicle protrusion from the seed coat. The adverse effects of both treatments were assessed at a physiological level by measuring the germination speed (T_{50}) and percentage (G%). Significant differences in T_{50} and G% values were found between the all investigated lentil populations in response to salinity. In general, we propose that salt stress delayed and decreased the germination in lentil seeds because of reducing of water uptake (osmotic effect) and/or accumulating high quantities of ions such as Na^+ and Cl^- (toxic effect). Although lentil is considered a very sensitive species to salinity, much more than other legumes such as broad bean, chickpea and soybean, the results achieved during germination experiments seem to suggest that the two lentil landraces (Capracotta and Conca Casale), Canadese and Turca Rossa varieties responded better to salt stress and may be utilized in breeding programs to improve the saline resistance in *Lens culinaris*.

Furthermore, this study provides the first proteome analysis of germinating lentil seeds in response to salt stress, and emphasizes the power and efficiency of a proteomic approach in discovering the proteins and mechanisms that are crucial for lentil stress response and tolerance.

Proteomic analysis was carried out by comparing protein profiles of the two Molise landraces and the five commercial varieties seeds germinating in presence of water (control) and 200 mM NaCl (stress). Analyses of variance (ANOVA) were performed using quantitative data of 339 differentially expressed protein spots in order to identify proteins which might have important roles in the salt stress response and to distinguish proteins that characterize the response to

salinity in Capracotta, Conca Casale and commercial varieties lentils. The identified proteins were involved in several processes, including storage, energy, disease/defence, cell structure, transport and protein synthesis. These results suggest that different lentil ecotypes may share a common pattern of response to salinity stress, but may have evolved also specific mechanisms resulting from their adaptation to specific growth environmental conditions.

Lastly, some proteins here reported might be indicated as potential candidates for enhancing salt tolerance in lentil and in crop by breeding and genetic engineering.

References

Abazarian, R., Yazdani, M.R., Khosroyar, K., Arvin, P. (2011) Effects of different levels of salinity on germination of four components of lentil cultivars. *African Journal of Agricultural Research*, **6**, 2761-2766.

Ackrell, B.A.C., Johnson, M.K., Gunsalus, R.P., Cecchini, G. (1992) Structure and function of succinate and fumarate reductase. In: *Chemistry and Biochemistry of Flavoenzymes* (ed. F. Muller) CRC Critical Reviews in Biochemistry, CRC Press, **3**, 229-297.

Aghaei, K., Ehsanpour, A.A., Shah, A.H., Komatsu, S. (2009). Proteome analysis of soybean hypocotyl and root under salt stress. *Amino Acids*, **36**, 91-98.

Agizzio, A.P., Carvalho, A.O., Ribeiro, S.F.F., Machado, O.L.T., Alves, E.W., Okorokov, L.A., Samaraò, S.S., Junior, C.B., Prates, M.V., Gomes, V.M. (2003) A 2S albumin-homologous protein from passion fruit seeds inhibits the fungal growth and acidification of the medium by *Fusarium oxysporum*. *Archives of Biochemistry and Biophysics*, **416**, 188-195.

Ahmad, P. & Jhon, R. (2005) Effect of salt stress on growth and biochemical parameters of *Pisum sativum* L. *Archives of Agronomy and Soil Science*, **51**, 665-672.

Al-Mutawa, M.M. (2003) Effect of salinity on germination and seedling growth of chickpea (*Cicer arietinum* L.) genotypes. *International Journal of Agriculture and Biology*, **3**, 226-229.

Almansouri, M., Kinet, J.M., Lutts, S. (2001) Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum* Desf.). *Plant and Soil*, **231**, 243-254.

Ali, G., Srivastava, P.S., Iqbal, M. (1999) Proline accumulation, protein pattern and photosynthesis in regenerants grown under NaCl stress. *Biologia Plantarum*, **42**, 89-95.

Almasouri, M., Kinet, J.M., Lutts, S. (1999) Compared effects of sudden and progressive imposition of salt stress in three durum wheat (*Triticum durum* Desf.) cultivars. *Journal of Plant Physiology*, **154**, 743-752.

Artlip, T.S. & Funkhouser, E.A. (1995) Protein synthetic responses to environmental stresses. In: *Handbook of Plant and Crop Physiology* (ed. M. Pessarakli) Marcel Dekker Inc., New York, 627-644.

Asgharipour, M.R. & Rafiei M. (2011) Effect of salinity on germination and seedling growth of lentils. *Australian Journal of Basic and Applied Sciences*, **5**, 2002-2004.

Ashraf, M. & Waheed, A. (1990) Screening of local/exotic accessions of lentil (*Lens culinaris* Medic.) for salt tolerance at two growth stages. *Plant and Soil*, **128**, 167-176.

Ashraf, M. & Waheed, A. (1993) Responses of some local/exotic accessions of lentil (*Lens culinaris* Medic.) to salt stress. *Journal of Agronomy and Crop Science*, **170**, 103-112.

Ashraf, M., Afaf, Y.R., Qureshi, M.S., Sarwar, G., Naqvi, M.H. (2002) Salinity induced changes in α-amylase and protease activities and associated metabolism in cotton varieties during germination and early seedling growth stages. *Acta Physiologiae Plantarum*, **24**, 37-44.

Ashraf, M. & Bashir, A. (2003) Salt stress induced changes in some organic metabolities and ionic relations in nodules and other plant parts of two crop legumes differing in salt tolerance. *Flora*, **198**, 486-498.

Ashraf, M. & Harris, P.J.C. (2004) Potential biochemical indicators of salinity tolerance in plants. Plant Science, **166**, 3-16.

Bandeoglu, E., Eyidogan, F., Yucel, M., Oktem, H.A. (2004) Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. *Plant Growth Regulation*, **42**, 69-77.

Babiychuk, E., Cottrill, P.B., Storozhenko, S., Fuangthong, M., Chen, Y., O'Farrell, M.K., Van Montagu, M., Inzé, D., Kushnir, S. (1998) Higher plants possess two structurally different poly(ADPribose) polymerases. *The Plant Journal: for Cell and Molecular Biology*, **15**, 635-645.

Bacchetta, G., Belletti, P., Brullo, S., Cagelli, L., Carasso, V., Casas, J.L., Cervelli, C., Escribà, M.C., Fenu, G., Gorian, F., Güemes, J., Mattana, E., Nepi, M., Pacini, E., Pavone, P., Piotto, B., Pontecorvo, C., Prada, A., Venora, G., Vietto, L., Virevaire, M. (2006) *Manuale per la raccolta, studio, conservazione e gestione ex situ del germoplasma*, Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici, Rome.

Bayuelo-Jimenez J.S., Graig, R., Lynch, J. (2002) Salinity tolerance of *Phaseolus* species during germination and early seedling growth. Seed Physiology, Production & Technology. *Crop Science*, **42**, 1584-1594.

Bayuelo-Jimenez, J.S., Debouck, D.G., Lynch, J.P. (2003) Growth, gas exchange, water relations, and ion composition of *Phaseolus* species grown under saline conditions. *Field Crops Research*, **80**, 207-222.

Ben-Hayyim, G. & Goffer, Y. (1989) Plantlet regeneration of NaCl-selected salt-tolerant callus culture of Shamouti orange (*Citrus sinensis* L. Osbeck). *Plant Cell Reports*, **7**, 680-683.

Bernstein, L. & Ogata, G. (1966) Effects of salinity on nodulation, nitrogen fixation, and growth of soybean and alfalfa. *Agronomy Journal*, **58**, 201-203.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., Drost, L., Ridley, P., Hudson, S.A., Patel, K., Murphy, G., Piffanelli, P., Wedler, H., Wedler, E., Wambutt, R., Weitzenegger, T., Pohl, T.M., Terryn, N., Gielen, J., Villarroel, R., De Clerck, R., Van Montagu, M., Lecharny, A., Auborg, S.,Gy, I., Kreis, M., Lao, N., Kavanagh, T., Hempel, S., Kotter, P., Entian, K.D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Silvey, M., James, R., Montfort, A., Pons, A., Puigdomenech, P., Douka, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, B., Hilbert, H., Düsterhöft, A., Moores, T., Jones, J.D., Eneva, T., Palme, K., Benes, V., Rechman, S., Ansorge, W., Cooke, R., Berger, C., Delseny, M., Voet, M., Volckaert, G., Mewes, H.W., Klosterman, S., Schueller, C., Chalwatzis, N. (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature*, **391**, 485-488.

Bliss, R.D., Plattaloia, K.A., Thomson, W.W. (1986) Osmotic sensitivity in relation to salt sensitivity in germinating barley seeds. *Plant Cell and Environment*, **9**, 721-725.

Blount, J.W., Dixon, R.A., Paiva, N.L. (1993) Stress responses in alfalfa (*Medicago sativa* L.) XVI. Antifungal activity of medicarpin and its biosynthetic precursors; implications for the genetic manipulation of stress metabolites. *Physiological and Molecular Plant Pathology*, **41**, 333-349.

Borowitzak, L.J. (1981) Solute accumulation and regulation of cell water activity. In: *The physiology and biochemistry of drought resistance in plants* (ed. L.G. Paleg & D. Aspinall). Academic Press, Sidney, 97-130.

Bourgeois, M., Jacquin, F., Savois, V., Sommerer, N., Labas, V., Henry, C., Burstin, J. (2009) Dissecting the proteome of pea mature seeds reveals the phenotypic plasticity of seed protein composition. *Proteomics*, **9**, 254-271.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248-254.

Brandalise, M., Severino, F.E., Maluf, M.P., Maia, I.G. (2009) The promoter of a gene encoding an isoflavone reductase-like protein in coffee (*Coffea arabica*) drives a stress-responsive expression in leaves. *Plant Cell Reports*, **28**, 1699-1708.

Braun, H., Czihal, A., Shutov, A.D., Bäumlein, H. (1996) A vicilin-like seed protein of cycads: similarity to sucrose binding proteins. *Plant Molecular Biology*, **31**, 35-44.

Bybordi, A. & Tabatabaei, J. (2009) Effect of salinity stress on germination and seedling properties in canola cultivars (*Brassica napus* L.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, **37**, 71-76.

Caruso, G., Cavaliere, C., Guarino, C., Gubbiotti, R., Foglia, P., Laganà, A. (2008) Identification of changes in *Triticum durum* L. leaf proteome in response to salt stress by two-dimensional electrophoresis and MALDI-TOF mass spectrometry. *Analytical and Bioanalytical Chemistry*, **391**, 381-390.

Casey, R., Downey, D., Ellis, N. (1986) Legume storage proteins and their genes. *Oxford Surveys of Plant Molecular and Cell Biology*, **3**, 1-95.

Castillo, J., Rodrigo, M.I., Márquez, J.A., Zúñiga, A., Franco, L. (2000) A pea nuclear protein that is induced by dehydration belongs to the vicilin superfamily. *European Journal of Biochemistry*, **267**, 2156-2165.

Castillo, J., Genovés, A., Franco, L., Rodrigo, M.I. (2005) A multifunctional bicupin serves as precursor for a chromosomal protein of *Pisum sativum* seeds. *Journal of Experimental Botany*, **56**, 3159-3169.

Chen, S., Gollop, N., Heuer, B. (2009) Proteomic analysis of saltstressed tomato (*Solanum lycopersicum*) seedlings: effect of genotype and exogenous application of glycinebetaine. *Journal of Experimental Botany*, **60**, 2005-2019.

Chiatante, D., Onelli, E., Patrignani, G., Scippa, G.S. (1995) Localization of a nuclear protein (QP47) in embryonic meristems during seed maturation and germination and its distribution among crop plants. *Journal of Experimental Botany*, **46**, 815-821.

Chitteti, B.R. & Peng, Z. (2007) Proteome and phosphoproteome differential expression under salinity stress in rice (*Oryza sativa*) roots. *Journal of Proteome Research*, **6**, 1718-1727.

Chung, R.P.T., Neumann, G.M., Polya, G.M. (1997) Purification and characterization of basic proteins with in vitro antifungal activity from seeds of cotton, *Gossypium hirsutum*. *Plant Science*, **127**, 1-16.

Contim, L.A., Waclawovsky, A.J., Delu-Filho, N., Pirovani, C.P., Clarindo, W.R., Loureiro, M.E., Carvalho, C.R., Fontes, E.P. (2003) The soybean sucrose binding protein gene family: genomic organization, gene copy number and tissue-specific expression of the SBP2 promoter. *Journal of Experimental Botany*, **54**, 2643-2653.

Cordovilla, M.P., Ligero, F., Lluch, C. (1995a) Influence of host genotypes on growth, symbiotic performance and nitrogen assimilation in faba bean (*Vicia faba* L.) under salt stress. *Plant and Soil*, **172**, 289-297.

Cordovilla, M.P., Ocana, A., Ligero, F., Lluch, C. (1995b) Salinity effects on growth analysis and nutrient composition in four grain legumes-*Rhizobium* symbiosis. *Journal of Plant Nutrition*, **18**, 1595-1600.

Cramer, G.R. (1992) Kinetics of maize leaf elongation. *Journal of Experimental Botany*, **43**, 857-864.

Dantas, B.F., Ribeiro, L.S., Aragao, C.A. (2007) Germination, initial growth and cotyledon protein content of bean cultivars under salinity stress. *Revista Brasileira de Sementes*, **29**, 106-110.

Day, I.S., Reddy, V.S., Shad Ali, G., Reddy, A.S. (2002) Analysis of EF-hand-containing proteins in Arabidopsis. *Genome Biology*, **3**, 1-24.

De Block, M., Verduyn, C., De Brouwer, D., Cornelissen, M. (2005) Poly(ADP-ribose) polymerase in plants affects energy homeostasis, cell death and stress tolerance. *The Plant Journal: for Cell and Molecular Biology*, **41**, 95-106.

de Lacerda, C.F., Cambraia, J., Oliva, M.A., Ruiz, H.A., Prisco, J.T. (2003) Solute accumulation and distribution during shoot and leaf development in two sorghum genotypes under salt stress. *Environmental and Experimental Botany*, **49**, 107-120.

Dell'Aquila, A. (2004) Protein patterns, characterized by computer image analysis, of lentil embryo axes germinating under salt stress. *Biologia Plantarum*, **28**, 237-242.

Delgado, M.J., Ligero, F., Lluch, C. (1994) Effects of salt stress on growth and nitrogen fixation by pea, faba-bean, common-bean, and soybean plants. *Soil Biology & Biochemistry*, **26**, 371-376.

Demir, Y. & Kocakalikan, I. (2002) Effect of NaCl and proline on bean seedlings cultured in vitro. *Biologia Plantarum*, **45**, 597-599.

Dixon, R.A. (2001) Natural products and plant disease resistance. *Nature*, **411**, 843-847.

Dkhil, B.B. & Denden, M. (2010) Salt stress induced changes in germination, sugars, starch and

enzyme of carbohydrate metabolism in *Abelmoschus esculentus* L. (Moench.) seeds. *African Journal of Agricultural Research*, **5**, 408-415.

Dodd, G.L. & Donovan, L.A. (1999) Water potential and ionic effects on germination and seedling growth of two cold desert shrubs. *American Journal of Botany*, **86**, 1146-1153.

Dooki, A.D., Mayer-Posner, F.J., Askari, H., Zaiee, A.A., Salekdeh, G.H. (2006) Proteomic responses of rice young panicles to salinity. *Proteomics*, **6**, 6498-6507.

Doucet-Chabeaud, G., Godon, C., Brutesco, C., de Murcia, G., Kazmaier, M. (2001) Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. *Molecular Genetics and Genomics*, **265**, 954-963.

Du, C.X., Fan, H.F., Guo, S.R., Tezuka, T., Li, J. (2010) Proteomic analysis of cucumber seedling roots subjected to salt stress. *Phytochemistry*, **71**, 1450-1459.

Dubey, R.S. (1983) Biochemical changes in germinating rice seeds under saline stress. *Biochemie und Physiologie der Pflanzen*, **177**, 523-535.

Dubey, R.S. (1985) Effect of salinity on nucleic acid metabolism of germinating rice seeds differing in salt tolerance. *Plant Physiology and Biochemistry*, **12**, 9-16.

Dubey, R.S. & Rani, M. (1987) Proteases and proteins in germinating rice seeds in relation to salt tolerance. *Plant Physiology and Biochemistry*, **14**, 174-182.

Dubey, R.S. & Rani, M (1989) Influence of NaCl salinity on growth and metabolic status of proteins and amino acids in rice seedlings. *Journal of Agronomy and Crop Science*, **162**, 97-106.

Dunwell, J.M., Khuri, S., Gane, P.J. (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. *Microbiology and Molecular Biology Reviews*, **64**, 153-179.

El-Hamdaoui, A., Redondo-Nieto, M., Torralba, B., Rivilla, R., Bonilla, I., Bolaños, L. (2003) Influence of boron and calcium on the tolerance to salinity of nitrogen-fixing pea plants. *Plant and Soil*, **251**, 93-103.

El-Monem A. & Sharaf M. (2008) Tolerance of five genotypes of lentil to NaCl-salinity stress. *New York Science Journal*, **1**, 70-80.

Elenany, A.E. (1997) Shoot regeneration and protein synthesis in tomato tissue cultures. *Biologia Plantarum*, **39**, 303-308.

Elmer, A., Chao, W., Grimes, H. (2003) Protein sorting and expression of a unique soybean cotyledon protein, GmSBP, destined for the protein storage vacuole. *Plant Molecular Biology*, **52**, 1089-1106.

Elsamad, H.M.A. & Shaddad, M.A.K. (1997) Salt tolerance of soybean cultivars. *Biologia Plantarum*, **39**, 263-269.

Elsheikh, E.A.E. & Wood, M. (1990) Effect of salinity on growth, nodulation and nitrogen yield of chickpea (*Cicer arietinum* L.). *Journal of Experimental Botany*, **41**, 1263-1269.

Esechie, H.A., Rodriguez, V., Al-Asmi, M.S. (2002) Effect of sodium chloride salinity on cation equilibria in alfalfa (*Medicago sativa* L.). *Crop Research*, **23**, 253-258.

Ferri, A., Lluch, C., Ocana, A. (2000) Effect of salt stress on carbon metabolism and bacteroid respiration in root nodules of common bean (*Phaseolus vulgaris*). *Plant Biology*, **2**, 396-402.

Flagella, Z., Trono, D., Pompa, M., Di Fonzo, N., Pastore, D. (2006) Seawater stress applied at germination affects mitochondrial function in durum wheat (*Triticum durum*) early seedlings. *Functional Plant Biology*, **33**, 357-366.

Francois, L.E., Donovan, T.J., Maas, E.V. (1990) Salinity effects on emergence, vegetative growth and seed yield of guar. *Agronomy Journal*, **82**, 587-591.

Gama, P., Inanaga, S., Tanaka, K., Nakazawa, R. (2007) Physiological response of common bean (*Phaseolus vulgaris* L.) seedlings to salinity stress. *African Journal of Biotechnology*, **6**, 79-88.

Gao, L.,Yan, X., Li, X., Guo, G., Hu, Y., Ma, W., Yan, Y. (2011) Proteome analysis of wheat leaf under salt stress by two-dimensional difference gel electrophoresis (2D-DIGE). *Phytochemistry*, **72**, 1180-1191.

Garg, B.K., Vyas, S.P., Kathju, S., Lahiri, A.N. (1998) Influence of water deficit stress at various growth stages on some enzymes of nitrogen metabolism and yield in clusterbean genotypes. *Indian Journal of Plant Physiology*, **3**, 214-218.

Ghassemi, F., Jakeman, A.J., Nix, H.A. (1995) *Salinisation of land and water resources: Human causes, extent management and case studies*, University of New South Wales Press Ltd, Sydney.

Gholipoor, M., Ghasemi-Golezani, K., Khooie, F.R., Moghaddam, M. (2000) Effects of salinity on initial seedling growth of chickpea (*Cicer arietinum* L.). *Acta Agronomica Hungarica*, **48**, 337-343.

Gietl, C. (1992) Malate dehydrogenase isoenzymes: cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles. *Biochimica et Biophysica Acta*, **1100**, 217-234.

Gomes, V.M., Mosqueda, M.I., Blanco-Labra, A., Sales, M.P., Fernandes, K.V.S., Cordeiro, R.A., Xavier-Filho, J. (1997) Vicilin storage proteins from *Vigna unguiculata* (legume) seeds inhibit fungal growth. *Journal of Agriculture and Food Chemistry*, **45**, 4110-4115.

Grattan, S.R. & Grieve, C.M. (1992) Mineral element acquisition and growth response of plants grown in saline environments. *Agriculture, Ecosystems & Environment*, **38**, 275-300.

Greenway, H. & Munns, R. (1980) Mechanisms of salt tolerance in nonhalophytes. *Annual Review of Plant Physiology*, **31**, 149-190.

Grimes, H.D., Overvoorde, P.J., Ripp, K., Franceschi, V.R., Hitz, W.D. (1992) A 62-kD sucrose binding protein is expressed and localized in tissues actively engaged in sucrose transport. *The Plant Cell*, **4**, 1561-1574.

Hadas, A. (1977) Water uptake and germination of leguminous seeds in soils of changing matrix and osmotic water potential. *Journal of Experimental Botany*, **28**, 977-985.

Hannaert, V., Henner, B., Ulrich, N., William, M. (2000) Enolase from *Trypanosoma brucei*, from the amitochondriate protist *Mastigamoeba balamuthi*, and from the chloroplast and cytosol of *Euglena gracilis*: pieces in the evolutionary puzzle of the eukaryotic glycolytic pathway. *Molecular Biology and Evolution*, **17**, 989-1000.

Hakim, M.A., Juraimi, A.S., Begum, M., Hanafi, M.M., Ismail, M.R., Selamat, A. (2010) Effect of salt stress on germination and early seedling growth of rice (*Oryza sativa* L.). *African Journal of Biotechnology*, **9**, 1911-1918.

Hampson, C.R. & Simpson, G,M. (1990) Effects of temperature, salt, and osmotic potential on early growth of wheat (*Triticum aestivum*). 1. Germination. *Canadian Journal of Botany*, **68**, 524-528.

Hasegawa, P. M., Bressan, R. A., Zhu, J. K., Bohnert, H. J. (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology*, ***51***, 463-499.

Haslekås, C., Viken, M,K., Grini, P.E., Nygaard, V., Nordgard, S.H., Meza, T.J., Aalen, R.B. (2003) Seed 1-cysteine peroxiredoxin antioxidants are not involved in dormancy, but contribute to inhibition of germination during stress. *Plant Physiology*, **133**, 1148-1157.

Heim, U., Wang, Q., Kurz, T., Borisjuk, L., Golombek, S., Neubohn, B., Adler, K., Gahrtz, M., Sauer, N., Weber, H., Wobus, U. (2001) Expression patterns and subcellular localization of a 55 kDa sucrose-binding protein homologue of *Vicia faba* (VfSBPL) suggest different functions during development. *Plant Molecular Biology*, **47**, 461-474.

Hernandez, J.A. & Almansa, M.S. (2002) Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. *Physiologia Plantarum*, **115**, 251-257.

Huang, J. & Redmann, R.E. (1995) Salt tolerance of *Hordeum* and *Brassica* species during germination and early seedling growth. *Canadian Journal of Plant Science*, **75**, 815-819.

Hurkman, W.J. & Tanaka, C.K. (1987) The effects of salt on the pattern of proteins synthesis in barley roots. *Plant Physiology*, **83**, 517-524.

Hurkman, W.J., Fornari, C.S., Tanaka, C.K. (1989) A comparison of the effect of salt on polypeptide and translatable mRNA in roots of a salt tolerant and salt sensitive cultivar of barley. *Plant Physiology*, **90**, 1444-1456.

Ialicicco, M., Viscosi, V., Scaloni, A., Arena, S., Trupiano, D., Rocco, M., Chiatante, D., Scippa, G.S. (2012) Seed proteome variation between two autochthon lentil (*Lens culinaris* Medik.) landraces. (*In preparation*).

Ivanishchev, V.V. (1997) Biological role of oxaloacetate metabolism in chloroplasts of C-3 plants. *Russian Journal of Plant Physiology*, **44**, 401-408.

Jain, M., Mathur, G., Koul, S., Sarin, N.B. (2001) Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.). Plant Cell Reports, **20**, 463-468.

Jamil, M. & Rha, E.S. (2004) The effect of salinity (NaCl) on the germination and seedling of sugar beet (*Beta vulgaris* L.) and cabbage (*Brassica oleracea* L.). *Korean Journal of Plant Resources*, **7**, 226-232.

Jamil, M., Lee, C.C., Rehman, S.U., Lee, D.B., Ashraf, M., Rha, E.S. (2005) Salinity (NaCl) tolerance of brassica species at germination and early seedling growth. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, **4**, 970-976.

Jang, H.J., Pih, K.T., Kang, S.G., Lim, J.H., Jin, J.B., Piao, H.L., Hwang, I. (1998) Molecular cloning of a novel Ca^{2+}-binding protein that is induced by NaCl stress. *Plant Molecular Biology*, **37**, 839-847.

Jebara, S., Jebara, M., Limam, F., Aouani, M.E. (2005) Changes in ascorbate peroxidase, catalase, guaiacol peroxidase and superoxide dismutase activities in common bean (*Phaseolus vulgaris*) nodules under salt stress. *Journal of Plant Physiology*, **162**, 929-936.

Jeong, M.J., Park, S.C., Byun, M.O. (2001) Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. *Molecules and Cells*, **12**, 185-189.

Jiang, Y., Yang, B., Harris, N.S., Deyholos, M.K. (2007) Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. *Journal of Experimental Botany*, **58**, 3591-3607.

Jin, H., Kim, H.R., Plaha, P., Liu, S.K., Park, J.Y., Piao, Y.Z., Yang, Z.H., Jiang, G.B., Kwak, S.S., Anf, G., Son, M., Jin, Y.H., Sohn, J.H., Lim, Y.P. (2008) Expression profiling of the genes induced by Na_2CO_3 and NaCl stresses in leaves and roots of *Leymus chinensis*. *Plant Science*, **175**, 784-792.

Jolivet, Y., Pireaux, J.C., Dizengremel, P. (1990) Changes in properties of barley leaf mitochondria isolated from NaCl-treated plants. *Plant Physiology*, **94**, 641-646.

Jones, R. A. (1986) High salt tolerance potential in *Lycopersicon* species during germination. *Euphytica*, **35**, 575-582.

Junghans, U., Polle, A., Düchting, P., Weiler, E., Kuhlmann, B., Gruber, F., Teichmann, T. (2006) Adaptation to high salinity in poplar involves changes in xylem anatomy and auxin physiology. *Plant, Cell and Environment*, **29**, 1519-1531.

Kafi, M. & Goldani, M. (2001) Effect of water potential and type of osmoticum on seed germination of three crop species of wheat, sugarbeet, and chickpea. *Agricultural Sciences and Technology*, **15**, 121-33.

Katerji, N., Hoorn, J.W., Hamdy, A., Mastrorilli, M., Oweis, T., Malhotra, R.S. (2001) Response to soil salinity of two chickpea varieties differing in drought tolerance. *Agricultural Water Management*, **50**, 83-96.

Kav, N.N.V., Srivastava, S., Goonewardene, L., Blade, S.F. (2004) Proteome-level changes in the roots of *Pisum sativum* in response to salinity. Annals of Applied Biology, **145**, 217-230.

Kaymakanova, M. (2009) Effect of salinity on germination and seed physiology in bean (*Phaseolus vulgare* L.). *Biotechnology & Biotechnological Equipment*, Special edition, **23**, 326-329.

Kim, S.T., Cho, K.S., Kim, S.G., Kang, S.Y., Kang, K.Y. (2003a) A rice isoflavone reductase-like gene, OsIRL, is induced by rice blast fungal elicitor. *Molecules and Cells*, **16**, 224-231.

Kim, S.T., Cho, K.S., Yu, S., Kim, S.G., Hong, J.C., Han, C.D., Bae, D.W., Nam, M.H., Kang, K.Y. (2003b) Proteomic analysis of differentially expressed proteins induced by rice blast fungus and elicitor in suspension-cultured rice cells. *Proteomics*, **3**, 2368-2378.

Kingstonsmith, A.H., Harbinson, J., Williams, J., Foyer, C.H. (1997) Effect of chilling on carbon assimilation, enzyme activation and photosynthetic electron transport in the absence of photoinhibition in maize leaves. *Plant Physiology*, **114**, 1039-1046.

Konishi, H., Kitano, H., Komatsu, S. (2005) Identification of rice root proteins regulated by gibberellin using proteome analysis. *Plant, Cell and Environment*, **28**, 328-339.

Kuhad, M.S. & Sheoran, I.S. (1987) Influence of simulated drought and salt stress at isoosmotic levels on seedling growth of pigeonpea genotypes. *International Pigeonpea Newsletter*, **6**, 48-50.

Kuriakose, S.V. & Prasad, M.N.V. (2007) Cadmium stress affects seed germination and seedling growth in *Sorghum bicolor* (L.) Moench by changing the activities of hydrolyzing enzymes. *Plant Growth Regulation*, **54**, 143-156.

Lauter, D.J., Munns, D.N., Clarkin, K.L. (1980) Salt response of chickpeas influenced by N supply. *Agronomy Journal*, **73**, 961-966.

Lers, A., Burd, S., Lomaniec, E., Droby, S., Chalutz, E. (1998) The expression of a grapefruit gene encoding an isoflavone reductase-like protein is induced in response to UV irradiation. *Plant Molecular Biology*, **36**, 847-856.

Levitt, J. (1972) *Responses of plants to environmental stresses*, Academic Press, London.

Lin, C.C. & Kao, C.H. (1995) NaCl stress in rice seedlings: starch mobilization and the influence of gibberellic acid on seedling growth. *Botanical Bulletin of Academia Sinica*, **36**, 169-173.

Ludwig-Müller, J. (2011) Auxin conjugates: their role for plant development and in the evolution of land plants. *Journal of Experimental Botany*, **62**, 1757-1773.

Luo, Q., Yu, B., Liu, Y. (2005) Differential sensitivity to chloride and sodium ions in seedlings of *Glycine max* and *G. soja* under NaCl stress. *Journal of Plant Physiology*, **162**, 1003-1012.

Ma, H., Song, L., Shu, Y., Wang, S., Niu, J., Wang, Z., Yu, T., Gu, W., Ma, H. (2012) Comparative proteomic analysis of seedling leaves of different salt tolerant soybean genotypes. *Journal of Proteomics*, **75**, 1529-1546.

Macedo, M.L.R., Andrade, L.B.S., Moraes, R.A., Xavier-Filho, J. (1993) Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comparative Biochemistry and Physiology*, **105**, 89-94.

Maghsoudi, M.A. & Maghsoudi, K. (2008) Salt stress effects on respiration and growth of germinated seeds of different wheat (Triticum aestivum L.) cultivars. *World Journal of Agricultural Sciences*, **4**, 351-358.

Mahajan, S. & Tuteja, N. (2005) Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics*, **444**, 139-158.

Mahajan, S., Pandey, G.K., Tuteja, N. (2008) Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. *Archives of Biochemistry and Biophysics*, **471**, 146-158.

Mahmoodzadeh, H. (2009) Protein profiles in response to salt stress in seeds of *Brassica napus*. *Research Journal of Environmental Sciences*, **3**, 225-231.

Mamo, T., Richter, C., Heiligtag, B. (1996) Salinity effects on the growth and ion contents of some chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medic) varieties. *Journal of Agronomy and Crop Science*, **176**, 235-247.

Manaa, A., Ben Ahmed, H., Valot, B., Bouchet, J.P., Aschi-Smiti, S., Causse, M., Faurobert, M. (2011) Salt and genotype impact on plant physiology and root proteome variations in tomato. *Journal of Experimental Botany*, **62**, 2797-2813.

Manchanda, G. & Garg, N. (2008) Salinity and its effects on the functional biology of legumes. *Acta Physiologiae Plantarum*, **30**, 595-618.

Mansour, M.M.F. (2000) Nitrogen containing compounds and adaptation of plants to salinity stress. *Biologia Plantarum*, **43**, 491-500.

Mehta, N.J. & Vora, A.B. (1987) Metabolic changes induced by NaCl salinity in pea plants. *International Conference of Plant Physiologists of SAARC Countries*, Gorakhpur, India, 47.

Mossad, M., Aleem M.A., Sabri S.R.S., Hanna N.S. (1992) Seedling characteristics as selection criteria for salinity tolerance in wheat. *Rachis*, **11**, 33-40.

Muccilli, V., Licciardello, C., Fontanini, D., Russo, M.P., Cunsolo, V., Saletti, R., Recupero, G.R., Foti, S. (2009) Proteome analysis of *Citrus sinensis* L. (Osbeck) flesh at ripening time. *Journal of Proteomics*, **73**, 134-152.

Munns, R. & Termaat, A. (1986) Whole plant responses to salinity. *Australian Journal of Plant Physiology*, **13**, 43-160.

Munns, R., Schachtman, D., Condon, A. (1995) The Significance of a Two-Phase Growth Response to Salinity in Wheat and Barley. *Functional Plant Biology*, **22**, 561-569.

Munns, R. (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment*, **25**, 239-250.

Munns, R. (2005) Genes and salt tolerance: bringing them together. *New Phytologist*, **167**, 645-663.

Munns, R. & Tester, M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, **59**, 651-681.

Murumkar, C.V. & Chavan, P.D. (1986) Influence of salt stress on biochemical processes in chickpea, *Cicer arietinum* L. *Plant and Soil*, **96**, 439-443.

Neumann, P.M., Van Volkenburgn, E., Cleland, R.E. (1988) Salinity stress inhibits bean leaf expansion by reducing turgor, not wall extensibility. *Plant Physiology*, **85**, 233-237.

Neumann, P. (1997) Salinity resistance and plant growth revisited. *Plant, Cell and Environment*, **20**, 1193-1198.

Nielsen, N.C., Dickinson, C.D., Cho, T.J., Thanh, V.H., Scallon, B.J., Fischer, R.L., Sims, T.L., Drews, G.N., Goldberg, R.B. (1989) Characterization of the glycinin gene family in soybean. *The Plant Cell*, **1**, 313-328.

Nieman, R.H. (1965) Expansion of bean leaves and its suppression by salinity. *Plant Physiology*, **40**, 156-161.

Nieman, R.H. & Maas, E.V. (1978) The energy change of salt stressed plants. *Sixth International Biophysics Congress Abstracts*, 121.

Nilsen, E.T. & Orcutt, D.M. (1996) *The physiology of plants under stress: Abiotic factors*, John Wiley & Sons, New York.

Nohzadeh Malakshah, S., Habibi Rezaei, M., Heidari, M., Salekdeh, G.H. (2007) Proteomic reveals new salt responsive proteins associated with rice plasma membrane. *Bioscience, Biotechnology, and Biochemistry*, **71**, 2144-2154.

Nonami, H., Tanimoto, K., Tabuchi, A., Fukwjama, T., Hashimoto, Y. (1995) Salt stress under hydroponic conditions causes changes in cell wall extension during growth. *Acta Horticulturae*, **396**, 91-98.

Okusanya, O.T. & Ungar, I.A. (1984) The growth and mineral composition of three species of spergularia as affected by salinity and nutrients at high salinity. *American Journal of Botany*, **71**, 439-447.

Oommen, A., Dixon, R.A., Paiva, N.L. (1994) The elicitor-inducible alfalfa isoflavone reductase promoter confers different patterns of developmental expression in homologous and heterologous transgenic plants. *The Plant Cell*, **6**, 1789-1803.

Overvoorde, P.J., Chao, W.S., Grimes, H.D. (1997) A plasma membrane sucrose-binding protein that mediates sucrose uptake shares structural and sequence similarity with seed storage proteins but remains functionally distinct. *The Journal of Biological Chemistry*, **272**, 15898-15904.

Pareek, A., Singla, S.L., Grover, A. (1997) Salt responsive proteins/genes in crop plants. In: *Strategies for improving salt tolerance in higher plants* (ed. P.K. Jaiwal, R.P. Singh, A. Gulati) Oxford and IBH Publication Co., New Delhi, 365-391.

Parker, R., Flowers, T.J., Moore, A.L., Harpham, N.V.J. (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. *Journal of Experimental Botany*, **57**, 1109-1118.

Petrucco, S., Bolchi, A., Foroni, C., Percudani, R., Rossi, G.L., Ottonello, S. (1996) A maize gene encoding an NADPH binding enzyme highly homologous to isoflavone reductases is activated in response to sulfur starvation. *The Plant Cell*, **8**, 69-80.

Pollard, T.D. & Cooper, J.A. (2009) Actin, a central player in cell shape and movement. *Science*, **326,** 1208-1212.

Popova, L.P., Stoinova, Z.G., Maslenkova, L.T. (1995) Involvement of abscisic acid in photosynthetic process in *Hordeum vulgare* L. during salinity stress. *Journal of Plant Growth Regulation*, **14**, 211-218.

Prado, F.E., Gonzalez, J.A., Boero, C., Gallardo, M., Boero, C., Kortsarz, A. (1995) Changes in soluble carbohydrates and invertase activity in *Chenopodium quinoa* developed for saline stress during germination. *Current Topics in Phytochemistry*, **14**, 1-5.

Qureshi, M.I., Qadir, S., Zolla, L. (2007) Proteomics-based dissection of stress-responsive pathways in plants. *Journal of Plant Physiology*, **164**, 1239-1260.

Rabilloud, T. (2000) *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods (Principles and Practice)*, Springer, Berlin Heidelberg, Germany.

Rengasamy, P. (2006) World salinization with emphasis on Australia. *Journal of Experimental Botany*, **57**, 1017-1023.

Riccardi, F., Gazeau, P., de Vienne, D., Zivy, M. (1998) Protein changes in response to progressive water deficit in maize. Quantitative variation and polypeptide identification. *Plant Physiology*, **117**, 1253-1263.

Richter, C., Heiligtag, B., Nagieb, M., Mukhallablati, U. (1999) The effects of salinity on growth and ion content of chickpea (*Cicer arietinum* L.), eggfruit (*Solanum melongena* L.) and red pepper (*Capsicum annuum* L.) varieties. *Tropenlandwirt*, **100**, 3-16.

Rocco, M., D'Ambrosio, C., Arena, S., Faurobert, M., Scaloni, A., Marra, M. (2006) Proteomic analysis of tomato fruits from two ecotypes during ripening. *Proteomics*, **6**, 3781-3791.

Saadallah, K,, Drevon, J., Mokhtar Abdelly, C. (2001) Genotypic variability for tolerance to salinity of N_2-fixing common bean (Phaseolus vulgaris). *Agronomie*, **21**, 675-682.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B., Bennett, J. (2002a) A proteomics approach to analyzing drought- and salt-responsiveness in rice. *Field Crops Research* ,**76**, 199-219.

Salekdeh, G.H., Siopongco, J., Wade, L.J., Ghareyazie, B., Bennett, J. (2002b) Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics*, **2**, 1131-1145.

Sales, M.P., Gerhardt, I.R., Grossi-De-Sá, M.F., Xavier-Filho, J. (2000) Do legume storage proteins play a role in defending seeds against bruchids? *Plant Physiology*, **124**, 515-22.

Sales, M.P., Pimenta, P.P., Paes, N.S., Grossi-de-Sa, M.F., Xavier-Filho, J. (2001) Vicilins (7S storage globulins) of cowpea (*Vigna unguiculata*) seeds bind to chitinous structures of the midgut of *Callosobruchus maculatus* (Coleoptera: Bruchidae) larvae. *Brazilian Journal of Medical and Biological Research*, **34**, 27-34.

Schaeffer, G.W., Sharpe, F.T., Sicher, R.C. (1997) Fructose 1,6-bisphosphate aldolase activity in leaves of a rice mutant selected for enhanced lysine. *Phytochemistry*, **46**, 1335-1338.

Scippa, G.S., Rocco, M., Ialicicco, M., Trupiano, D., Viscosi, V., Di Michele, M., Arena, S., Chiatante, D., Scaloni, A. (2010) The proteome of lentil (*Lens culinaris* Medik.) seeds: discriminating between landraces. *Electrophoresis*, **31**, 497-506.

Scippa, G.S., Petrollini, E., Trupiano, D., Rocco, M., Falco, G., Di Michele, M., Chiatante, D. (2011) Dormancy of *Medicago marina* (L.) seed. *Environmental and Experimental Botany*, **72**, 320-329.

Sekeroglu, N., Kara, M.S., Dede, O., Askin, T. (1999) Effect of salinity on germination, early seedling growth, Na and K constituents of chickpea. *Turkish Journal of Field Crops*, **4**, 79-84.

Seki, M., Kamei, A., Yamaguchi-Shinozaki, K., Shinozaki, K. (2003a) Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Current Opinion in Biotechnology*, **14**, 194-199.

Seki, M., Kamei, A., Satou, M., Sakurai, T., Fujita, M., Oono, Y., Yamaguch-Shinozaki, K., Shinozaki, K. (2003b) Transcriptome analysis in abiotic stress conditions in higher plants. *Topics in Current Genetics*, **4**, 271-295.

Semighini, C.P., Savoldi, M., Goldman, G.H., Harris, S.D. (2006) Functional characterization of the putative *Aspergillus nidulans* poly(ADP-ribose) polymerase homolog PrpA. *Genetics*, **173**, 87-98.

Serraj, R., Vasquez-Diaz, H., Hernandez, G., Drevon, J.J. (2001) Genotypic difference in the short-term response of nitrogenase activity (C_2H_2 reduction) to salinity and oxygen in the common bean. *Agronomie*, **21**, 645-651.

Shalhevet, J., Huck, M.G., Schroeder, B.P. (1995) Root and shoot growth responses to salinity in maize and soybean. *Agronomy Journal*, **87**, 512-516.

Sharma, K.D., Datta, K.S., Varma, S.K. (1990) Effect of chloride and sulphate salinity on nodulation and nitrogen fixation in chickpea. *Annals of Biology*, **6**, 3-20.

Shewry, P.R., Napier, J.N., Tatham, A.S. (1995) Seed storage proteins: structures and biosynthesis. *The Plant Cell*, **7**, 945-956.

Shoji, T., Winz, R., Iwase, T., Nakajima, K., Yamada, Y., Hashimoto, T. (2002) Expression patterns of two tobacco isoflavone reductaselike genes and their possible roles in secondary metabolism in tobacco. *Plant Molecular Biology*, **50**, 427-440.

Sibole, J.V., Montero, E., Cabot, C., Poschenrieder, C., Barcelo, J. (1998) Role of sodium in the ABA-mediated long-term growth response of bean to salt stress. *Physiologia Plantarum*, **104**, 299-305.

Sidari, M., Santonoceto, C., Anastasi, U., Preiti, G., Muscolo, A. (2008) Variations in four genotypes of lentil under NaCl-salinity stress. *American Journal of Agricultural and Biological Science*, **3**, 410-416.

Singh, K.N., Chatrath, R. (2001) Salinity Tolerance. In: *Application of physiology in wheat breeding* (ed. M.P. Reynolds, J.I.O. Monasterio, A. McNab) CIMMYT, Mexico, DF, 101-110.

Singh, A., Kumari, R., Garg, A.P., Varma, A. (2005) Endomycorrhizal diversity. In: *Basic research and applications of mycorrhizae* (ed. G.K. Podila & A. Varma) IK International, New Delhi, 1-23.

Singla, R. & Garg, N. (2005) Influence of salinity on growth and yield attributes in chickpea cultivars. *Turkish Journal of Agriculture and Forestry*, **29**, 231-235.

Smith, P.T. & Comb, B.G. (1991) Physiological and enzymatic activity of pepper seeds (*Capsicum annuum*) during priming. *Physiologia Plantarum*, **82**, 433-439.

Sobhanian, H., Razavizadeh, R., Nanjo, Y., Ehsanpour, A.A., Rastgar Jazii, F., Motamed, N., Komatsu, S. (2010a) Proteome analysis of soybean leaves, hypocotyls and roots under salt stress. *Proteome Science*, **8**, 1-15.

Sobhanian, H., Motamed, N., Rastgar Jazii, F., Nakamura, T., Komatsu, S. (2010b) Salt stress induced differential proteome and metabolome response in the shoots of *Aeluropus lagopoides* (Poaceae), a Halophyte C_4 Plant. *Journal of Proteome Research*, **9**, 2882-2897.

Sobhanian, H., Aghaei, K., Komatsu, S. (2011) Changes in the plant proteome resulting from salt stress: toward the creation of salt-tolerant crops? *Journal of Proteomics*, **74**, 1323-1337.

Sousa, M.F., Campos, F.A.P., Prisco, J.T., Eneas-Filho, J., Gomes-Filho, E. (2003) Growth and protein pattern in cowpea seedlings subjected to salinity. *Biologia Plantarum*, **47**, 341-346.

Soussi, M., Ocana, A., Lluch, C. (1998) Effects of salt stress on growth, photosynthesis and nitrogen fixation in chick-pea (*Cicer arietinum* L.). *Journal of Experimental Botany*, **49**, 1329-1337.

Soussi, M., Lluch, C., Ocana, A. (1999) Comparative study of nitrogen fixation and carbon metabolism in two chickpea (*Cicer arietinum* L.) cultivars under salt stress. *Journal of Experimental Botany*, **50**, 1701-1708.

Suzuki, N., Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Mittler, R. (2005) Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. *Plant Physiology*, **39**, 1313-1322.

Talamo, F., D'Ambrosio, C., Arena, S., Del Vecchio, P., Ledda, L., Zehender, G., Ferrara, L., Scaloni, A. (2003) Proteins from bovine tissue and biological fluids: Defining a reference electrophoresis map of liver, kidney, muscle, plasma and red blood cells. *Proteomics*, **3**, 440-460.

Trono, D., Flagella, Z., Laus, M.N., Di Fonzo, N., Pastore, D. (2004) The uncoupling protein and the potassium channel are activated by hyperosmotic stress in mitochondria from durum wheat seedlings. *Plant, Cell and Environment*, **27**, 437-448.

Umeda, M., Hara, C., Matsubayashi, Y., Li, H.H., Liu, Q., Tadokoro, F., Aotsuka, S., Uchimiya, H. (1994) Expressed sequence tags from cultured cells of rice (*Oryza sativa* L.) under stressed conditions: analysis of genes engaged in ATP-generating pathways. *Plant Molecular Biology*, **25**, 469-478.

Van der Straeten, D., Rodrigues-Pousada, R.A., Goodman, H.M., Van Montagu, M. (1991) Plant enolase: gene structure, expression, and evolution. *The Plant Cell*, **3**, 719-735.

Venema, J. &Tollervey, D. (1999) Ribosome synthesis in *Saccharomyces cerevisiae*. *Annual review of Genetics*, **33**, 261-311.

Walz, A., Park, S., Slovin, J.P., Ludwig-Müller, J., Momonoki, Y., Cohen, J.D. (2002) A gene encoding a protein modified by the phytohormone indoleacetic acid. *Proceedings of the National Academy of Science*, USA, **99**, 1718-1723.

Walz, A., Seidel, C., Rusak, G., Park, S., Cohen, J.D., Ludwig-Müller, J. (2008) Heterologous expression of IAP1, a seed protein from bean modified by indole-3-acetic acid, in *Arabidopsis thaliana* and *Medicago truncatula*. *Planta*, **227**, 1047-1061.

Wang, W., Vinocur, B., Altman, A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta,* **218**, 1-14.

Wang, J., Suen, P.K., Xu, Z.F., Jiang, L. (2009) A 64 kDa sucrose binding protein is membrane-associated and tonoplast-localized in developingmung bean seeds. *Journal of Experimental Botany*, **60**, 629-639.

Wang, C., Zhang, L.J., Huang, R.D. (2011) Cytoskeleton and plant salt stress tolerance. *Plant Signaling and Behavior*, **6**, 29-31.

Weimberg, R. (1967) Effect of sodium chloride on the activity of a soluble malate deyhdrogenase from pea seeds. *Journal of Biological Chemistry*, **242**, 3000-3006.

Welfare, K., Yeo, A.R., Flowers, T.J. (2002) Effect of salinity and ozone, individually and in combination on the growth and ion contents of two chickpea (*Cicer arietinum* L.) varieties. *Environmental Pollution*, **120**, 397-403.

Witzel, K., Weidner, A., Surabhi, GK., Börner, A., Mock, H.P. (2009) Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. *Journal of Experimental Botany*, **60**, 3545-3557.

Wright, R., Basson, M., D'Ari, L., Rine, J. (1988) Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. *Journal of Cell Biology*, **107**, 101-14.

Wyn Jones, R.G. & Pollard, A. (1983) Proteins, enzymes and inorganic ions. In: *Encyclopedia of plant physiology. Inorganic Plant Nutrition* (ed. A. Lauchli & R.L. Bieleski) Springer Verlag, Heidelberg, 528-562.

Xu, X.Y., Fan, R., Zheng, R., Li, C.M., Yu, D.Y. (2011) Proteomic analysis of seed germination under salt stress in soybeans. *Journal of Zhejiang University. Science*, **12**, 507-517.

Yan, S., Tang, Z., Su, W., Sun, W. (2005) Proteomic analysis of salt stress-responsive proteins in rice root. *Proteomics*, **5**, 235-244.

Younis, S.A., Shahatha, H.A., Hagop, P.G., Al-Rawi, F.I. (1991) Effect of salinity on the viability of rice seeds. In: *Plant Growth, Drought and Salinity in the Arab Region* (ed. K.H. Batanouny) The Egyptian Botanical Society, 235-244.

Yunes, A.N.A., Andrade, M.T., Sales, M.P., Morais, R.A., Fernandes, K.V.S., Gomes, V.M., Xavier-Filho, J. (1998) Legume seed vicilins (7S storage proteins) interfere with the development of the cowpea weevil (*Callosobruchus maculatus* (F)). *Journal of the Science of Food and Agriculture*, **76**, 111-116.

Zaccardelli, M., Lupo, F., Piergiovanni, A.R., Laghetti, G., Sonnante, G., Daminati, M.G., Sparvoli, F., Lioi, L. (2011) Characterization of Italian lentil (*Lens culinaris* Medik.) germplasm by agronomic traits, biochemical and molecular markers. *Genetic Resources and Crop Evolution*, 1-12.

Zhang, J.Z., Creelman, R.A., Zhu, J.K. (2004) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. *Plant Physiology*, **135**, 615-621.

Zhang, L., Yu, Z.F., Jiang, L., Jiang, J., Luo, H.B., Fu, L.R. (2011) Effect of post-harvest heat treatment on proteome change of peach fruit during ripening. *Journal of Proteomics*, **74**, 1135-1149.

Zhao, J., Zuo, K., Tang, K. (2004) cDNA cloning and characterization of enolase from *Chinese cabbage*, *Brassica campestris* ssp. Pekinensis. *DNA Sequence: The Journal of DNA Sequencing and Mapping*, **15**, 51-57.

Zhu, J.K. (2007) Plant salt stress. In: *Encyclopedia of Life Sciences* (ed. John Wiley & Sons), Chichester.

Zörb, C., Schmitt, S., Neeb, A., Karl, S., Linder, M., Schubert, S., (2004) The biochemical reaction of maize (*Zea mays* L.) to salt stress is characterized by a mitigation of symptoms and not by a specific adaptation. *Plant Science*, **167**, 91-100.

Chapter III

Novel application of isothermal DNA amplification for SSR genotyping.

This chapter reports the study concerning the "Novel application of isothermal DNA amplification for SSR genotyping". The researches activities, summarized in a paper (in preparation), have been focused on the development of a new molecular approach for genotyping and characterization of local varieties.

Although its main application is the detection for the presence/absence of target DNA, we applied the loop-mediated isothermal DNA amplification (LAMP) method to amplify a rice SSR. The results obtained by the LAMP method completely agreed with those obtained by polymerase chain reaction (PCR) analysis. Based on these results, LAMP is an alternative, rapid and simple method that may be useful for SSR genotyping.

The work was carried on at the National Institute of Agricultural Botany (NIAB) and was supervised by Dr. David Lee.

INTRODUCTION

3.1. Loop-mediated Isothermal Amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is a powerful innovative gene amplification technique and can amplify DNA with high specificity, efficiency and rapidity under isothermal condition (Notomi et al., 2000). Unlike PCR, a denatured template is not required (Nagamine et al., 2001) and DNA is generated in large amounts in a short time and positive LAMP reactions can be visualized with the naked eye (Mori et al., 2001). The main advantage of this technique is its simplicity; simple and cheap equipment, like a water bath or heating block, is needed to provide a constant temperature as the amplification proceeds under isothermal conditions.

The LAMP method requires a set of four different primers specifically designed to recognize six distinct regions on the target DNA. An inner primer with sequences of sense and anti-sense strands of the target initiates LAMP. A pair of 'outer' primers then displaces the amplified strand with the help of *Bst* DNA polymerase which has a high displacement activity, to release a single stranded DNA, which then forms a hairpin to initiate the starting loop for cyclic amplification.

The final products are stem loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops due to hybridization between alternately inverted repeats in the same strand (Notomi et al., 2000).

In 2002, Nagamine and coworkers improved this method by adding two additional primers, named loop primers. The loop primers hybridized to the stem-loops structures reducing reaction time to less than half of the original LAMP method (Nagamine et al., 2002).

3.2 Design of LAMP primers

Design of a highly sensitive and specific primer set is crucial for performing LAMP amplification.

A set of six primers comprising two outer, two internal and two loop primers that recognize eight distinct regions on the target sequence, is required for LAMP amplification. The two outer primers are known as forward outer primer (F3) and backward outer primer (B3) and have a role in strand displacement during the non-cyclic step only. The inner primers were described as forward internal primer (FIP) and backward internal primer (BIP) having both sense and antisense sequence in such a way that it helps in the formation of a loop. Further, forward loop primer (FLP) and backward loop primer (BLP) were designed to accelerate the amplification reaction by binding to additional sites that are not accessed by internal primers.

The design of the above mentioned six types of primers are based on the following eight distinct regions of the target gene: the F3c, F2c, F1c and FLP regions at the 3' side and the B1, B2, B3 and BLP regions at the 5' side (Figure 1) (Parida et al., 2008).

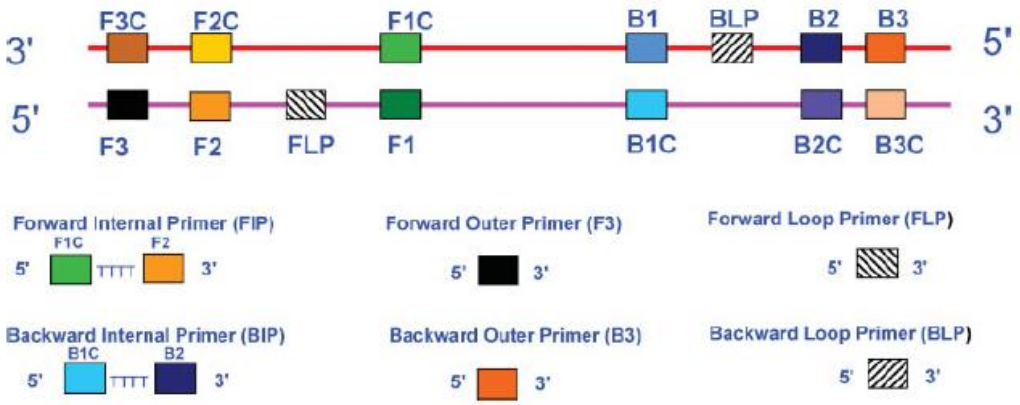

Figure 1. Schematic representation of loop-mediated isothermal amplification (LAMP) primers showing their position spanning the target gene. The inner primers FIB (BIP) are composed of F2 (B2) and F1c (B1c). The outer primers are designed at the region of F3 and B3. The loop primers are designed between F1c (B1c) and F2c (B2c) (original image in Parida et al., 2008).

FIP consists of the F2 region that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end. Forward outer primer (F3) consists of the F3 region that is complementary to the F3c region. BIP consists of the B2 region that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. Backward outer primer (B3) consists of the B3 region that is complementary to the B3c region. FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2 (Figure 1) (Notomi et al., 2000). The FLP and BLP primers were composed of the sequences that are complementary to the sequence between F1&F2 and B1&B2 regions respectively (Nagamine et al., 2002).

The size and sequence of the primers were chosen so that their melting temperature (T_m) is between 60-65 °C, the optimal temperature for *Bst* polymerase. The F1c and B1c T_m values should be a little higher than those of F2 and B2 to form the looped out structure. The T_m values of the outer primers F3 and B3 have to be lower than those of F2 and B2 to assure that the inner primers start synthesis earlier than the outer primers. Additionally, the concentrations of the inner primers are higher than the concentrations of the outer primers (Notomi et al., 2000).

3.3 Principle of LAMP amplification

LAMP amplification is based on the principle of auto cyclic strand displacement. The mechanism of the LAMP amplification reaction includes two steps: non-cyclic and cyclic step (Notomi et al., 2000; Parida et al., 2008) (Figure 2).

The non-cyclic step starts when inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis. The F3 primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-

linked complementary strand. A double strand is formed from the DNA strand synthesized from the F3 primer and the template DNA strand. The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions. This single strand DNA in turn serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced by the above step. Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 primer. The BIP-linked complementary strand displaced forms a structure with stem-loops at each end, which looks like a dumbbell structure. This dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. This structure serves as the starting material for LAMP cycling the second stage of the LAMP reaction.

During cycling amplification one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. Briefly the FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand. The released single strand then forms a dumbbell-like structure as both ends have complementary F1-F1c and B1c-B1 regions, respectively. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed. The final amplified products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Notomi et al., 2000; Nagamine et al., 2002; Tomita et al., 2008). The cycling reaction occurs in less than an hour with accumulation of 10^9 copies of the target (Notomi et al., 2000).

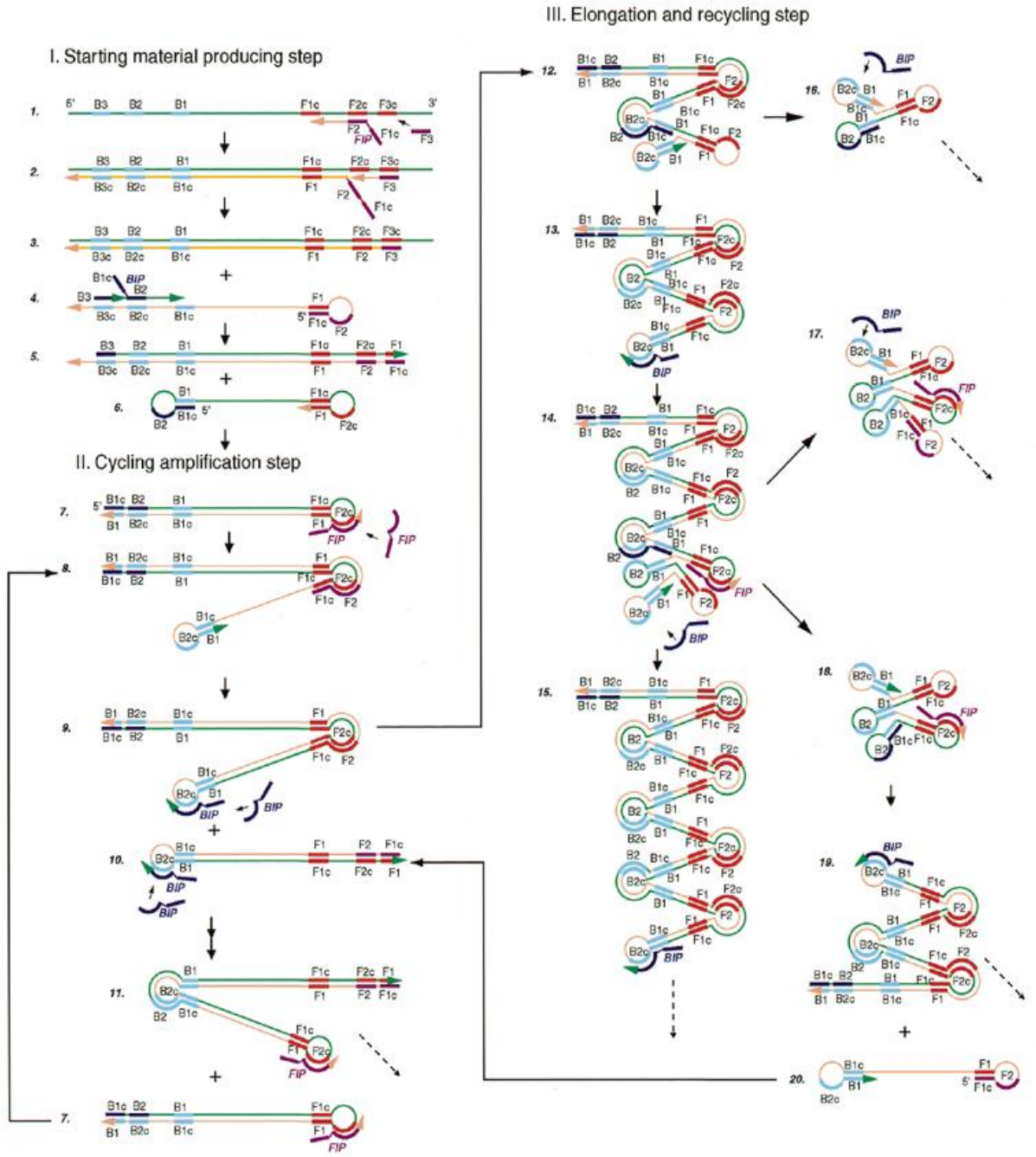

Figure 2. Schematic representation of loop-mediated isothermal amplification (LAMP) mechanism. In first step, in which starting material is produced, the dumbbell-like DNA form is generated (structure 6). Then in cycling amplification step, DNAs of this form are generated continuously. The elongation reactions are started from the sub-products (structures 10 and 11) of the cycling amplification step, generating various sizes of the products (original image in Notomi et al., 2000).

LAMP amplification can also be accomplished with the two outer (F3 and B3) and two internal primers (FIP and BIP) but by using the two loop primers (FLP and BLP), the amplification is accelerated thereby reducing the amplification time. The time required for amplification with loop primers is one-third to one-half of that without loop primer. With the use of loop primers, amplification can be achieved within 30 min (Nagamine et al., 2002) (Figure 3).

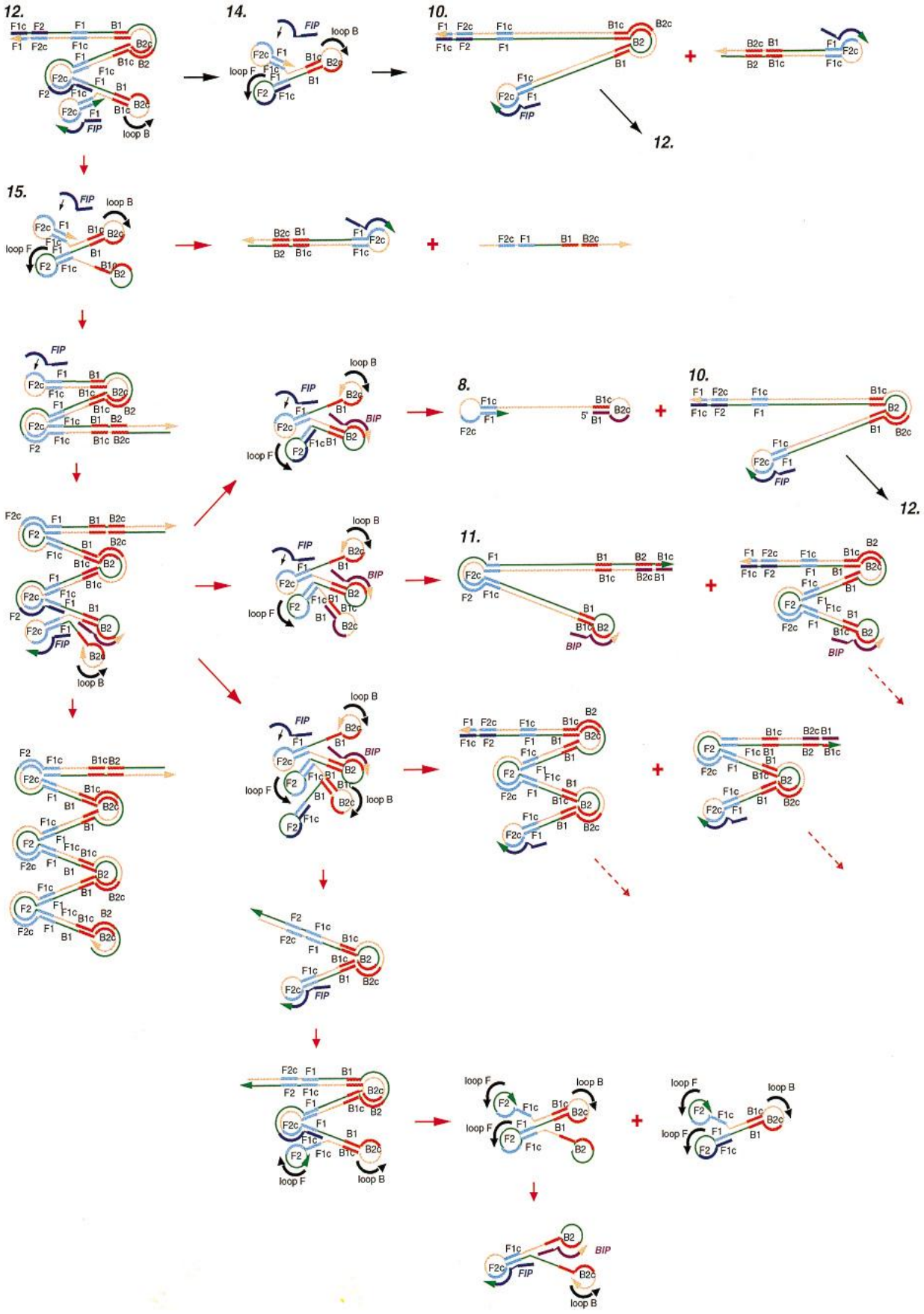

Figure 3. This figure shows the process that starts from structure 12. Black arrows show the original LAMP reaction pathway. Red arrows show the reaction derived from the loop primers. Red dotted arrows represent continuous reactions. The region between F1 and F2 (or B1c and B2c) is hybridized by the loop primer (original image in Nagamine et al., 2002).

3.4 Advantages of LAMP

LAMP has many unique advantages for which it holds great promise to be a method of choices for nucleic acid amplification (Fakruddin, 2011).

The primary characteristic of LAMP is its ability to amplify nucleic acid under isothermal conditions in the range of 65°C, allowing the use of simple cost effective reaction equipments (Parida et al., 2008). The second characteristic is that both amplification and detection of nucleic acid sequences can be completed in single step by incubating the mixture of sample, primers, *Bst* DNA polymerase at a constant temperature (Notomi et al., 2000).

Moreover, the LAMP assay has high specificity, because the amplification reaction occurs only when all six or eight regions within a target DNA are correctly recognized by the primers (Mori & Notomi, 2009).

In addition the amplification efficiency of LAMP is very high and the reaction proceeds rapidly since it does not require thermal cycling because of its isothermal reaction (Nagamine et al., 2001).

One of the most important advantages of LAMP is that large amounts of DNA are generated in a short time increasing the concentration of pyrophosphate ions. The produced turbidity observed as a white precipitate enables visual detection of positive LAMP reactions (Mori et al., 2001) and reduces time lost in post amplification analysis.

Another important advantage of Lamp is its ability to amplify the target from crude biological materials (Iwasaki et al., 2003; Poo et al., 2005; Lee et al., 2009b), contributing to saving the time and cost required for sample processing steps (Mori & Notomi, 2009). Finally, the Lamp method is characterized by high sensitivity, requiring only a few copies of the target for amplification and detection (Lee et al., 2009a).

References

Fakruddin, M. (2011) Loop mediated Isothermal Amplification (LAMP) - An Alternative to Polymerase Chain Reaction (PCR). *Bangladesh Research Publications Journal*, **5**, 425-439.

Iwasaki, M., Yonekawa, T., Otuska, K., Suzuki, W., Nagamine, K., Hase, K., Horigome, T., Notomi, T., Kanda, H. (2003) Validation of the Loop-mediated Isothermal Amplification Method for Single Nucleotide Polymorphism Genotyping with Whole Blood. *Genome Letters*, **2**,119-126.

Lee, D., La Mura, M., Allnutt, T.R., Powell,W. (2009a) Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. *BMC Biotechnology*, **9**, 7.

Lee, D., La Mura, M., Allnutt, T., Powell, W., Greenland, A. (2009b) Isothermal amplification of GM DNA sequences directly from plant tissues lowers the barriers to high-throughput and field-based genotyping. *Journal of Agricultural and Food Chemistry*, **57**, 9400-9402.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T. (2001) Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications*, **289**, 150-154.

Mori, Y. & Notomi, T. (2009) Loop-mediated isothermal amplification (LAMP): A rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infection and Chemotherapy*, **15**, 62-69.

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., Notomi, T. (2001) Loop-mediated isothermal amplification reaction using a nondenaturated template. *Clinical Chemistry*, **47**, 1742-1743.

Nagamine, K., Hase, T., Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, **16**, 223-229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**, E63.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L., Morita, K. (2008) Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*, **18**, 407-421.

Poon, L.L.M., Wong, B.W.Y., Ma, E.H.T., Chan, K.H., Chow, L.M.C., Abeyewickreme, W., Tangpukdee, N., Yuen, K.Y., Guan, Y., Looareesuwan, S., Peiris, J.S. (2006) Sensitive and inexpensive molecular test for Falciparum malaria: detecting *Plasmodium falciparum* DNA

directly from heat-treated blood by loop-mediated isothermal amplification. Clinical Chemistry, **52**, 303-306.

Tomita, N., Mori, Y., Kanda, H., Notomi, T. (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protocols*, **3**, 877-882.

(Paper in preparation)

Genotyping SSRs by isothermal DNA amplification of length variants

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Key words: Loop-mediated isothermal DNA amplification (LAMP), rice SSR, PCR

Abstract

Isothermal DNA amplification can be performed without the need for complex thermocyclers. Here we demonstrate the novel use of the loop-mediated isothermal DNA amplification method to amplify a rice SSR and show results to be consistent with analysis performed by PCR. We test the sensitivity of the assay and show it to amplify from near single copy target.

1. Introduction

Since its invention, polymerase chain reaction (PCR) (Saiki et al., 1985) has dominated the molecular biological research landscape on how genetic analysis is performed. At its core is the primer-directed DNA amplification of target sequence using cycles of denaturation, primer annealing and primer extension steps to replicate the desired sequence. That the products act as potential template for further replication is key to the amplification process. The enzyme most often used for PCR is *Taq* polymerase (*Taq* pol), which is thermostable and derived from the thermophilic bacterium *Thermophilus aquaticus*.

In PCR, templates are recycled for more amplification by denaturation. The need to use high temperatures has a negative impact on the longevity of *Taq* pol. More importantly, it appears to make the enzyme more prone to inhibitors found in biological samples. DNA amplification methods that utilize single temperature regimes (isothermal) are more robust than PCR and are

capable of amplifying from fairly crude DNA templates (Hosono et al., 2003; Iwasaki et al., 2003; Mitani et al., 2007; Lee et al., 2009c). Likewise, 'isothermal PCR', where a helicase unwinds the two strands allowing *Taq* pol to make more copies of the target with denaturation, is able to amplify from crude samples (Vincent et al., 2004).

Prior to the use of helicases, isothermal reactions cannot denature template and rely on the 5'-3' strand displacement properties of the polymerase, to make available single stranded DNA for further replication. This requirement has therefore restricted isothermal amplification, until recently, to the use of polymerases with strand displacement activities (e.g. *Bst* and phi29 polymerases).

The isothermal amplification of specific sequences can be achieved by circularisation of target and replication by rolling circle amplification (RCA) which produces tandemly repeating units of the circular sequence (Fire & Xu, 1995). This has been exploited in padlock probe genotyping (Nilsson et al., 1994). As an alternative method to exploit primer directed DNA amplification in an isothermal regime, loop mediated isothermal DNA amplification (LAMP) (Notomi et al., 2000; Nagamine et al., 2002) uses primers that contain sequences on the target that is 3' to the target annealing site. Once DNA synthesis from the primer goes beyond the 'common' sequence (present both on target and primer) the new DNA fragment contains both the common sequence and its reverse complement. This allows stem loop formation via *intra*-molecular hybridisation of the two sequences. Stem loops at each end of a DNA molecule produce a dumbbell structure that is capable of autocycling of template by strand displacement and primer annealing to generate an amplification reaction that produces a multimeric series of repeating inverted repeats. LAMP has been shown to be robust and highly specific with near single copy sensitivity (Lee et al., 2009a; Lee et al., 2009c). Although its main application is the detection for the presence/absence of target DNA, the assay can be modified to differentiate between single nucleotide variants for genotyping (Iwasaki et al., 2003). Here we have incorporated a rice simple sequence repeat (SSR) motif within a LAMP assay and show that length variants amplified were consistent with results obtained by PCR. Even though we exceeded the recommended limit for LAMP targets, we find the amplification of the SSR motifs to be highly sensitive with near-single copy detection.

2. Materials and Methods

Rice varieties used in this study are listed in Table 1. Rice DNA used was obtained from two sources: some samples were obtained from the EURIGEN collection (www.eurigen.net). The varieties LTH and SHZ and their inter-specific hybrid have been described previously (Akkinepalli et al., submitted).

Accession	Species
Augusto	Oryza sativa ssp. japonica
Augusto Red	O. sativa ssp. japonica
Ermes	O. sativa ssp. japonica
Venere	O. sativa ssp. japonica
Vialone Red	O. sativa ssp. japonica
LTH	O. sativa ssp. japonica
SHZ	O. sativa ssp. indica
F1 (LTH x SHZ)	Interspecific hybrid

Table 1. Rice DNA samples used in this study.

Primers were designed to amplify the rice SSR locus RM5436 (http://www.gramene.org/markers/microsat/ssr.html) using LAMP using parameters described previously (Lee et al., 2009a). Reactions were performed using a commercial LAMP amplification mastermix as recommended by the supplier (Optigene, Horsham, West Sussex). Table 2 lists the primers (Sigma-Genosys, Haverhill, Suffolk) used in the assays. SSR amplified products were denatured and loaded on an ABI3730 genotyper as described by Jones et al. (2011).

Number	Primer name	Sequence
1	displ_f	ATAGTTAAGCGGTGTGC
2	lamp_f	CTCGTCCTACATGTGCTTTTCAATCAAATCATCAACTGCTG
3	loop_f	*GAACAGGATGGACTGG
4	loop_r	GTCAACTGTTTCATATATC
5	lamp_r	ATGTCTCATGAGGGCCTTTTTTGGCTAAAGATACAAGGC
6	displ_r	CAATCCAGGTAGGCAG

Table 2. Primer names and sequences used in this study. The numbers denoted to the primers allow visualisation of their position in Figure 1. The asterisk denotes the FAM-labelled primers used to visualise products in an automated genotyper.

3. Results and Discussion

The amplification of an SSR motif using LAMP has not been previously reported. This may be due in part due to the size limitation warnings given by the inventors of LAMP. With six primers hybridising to eight regions within the target it is difficult to constrain an SSR-containing fragment to less than 200 bp limit suggested (Notomi et al., 2000; Tomita et al., 2008). For this experiment we tried to minimise the fragment size as best we could whilst incorporating the SSR.

The systems required to differentiate between SSR alleles require single nucleotide resolution so we incorporated a fluorescently labelled primer within the assay. The SSR motifs were positioned between the loop and lamp primers (Figure 1). This makes it possible to label the

SSR-containing fragments using a modified loop primer adjacent to the SSR motif. Even though it is possible to label SSR fragments by labelling the LAMP primers, the loop primer was chosen as it is shorter than the lamp primers and loop-lamp products are smaller than the alternative lamp-lamp products.

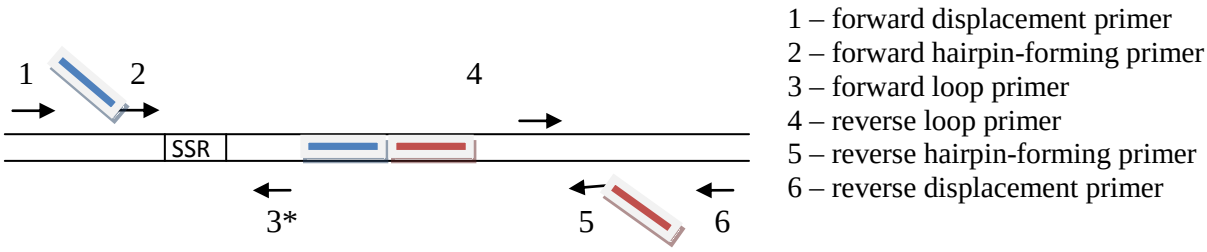

Figure 1. SSR genotyping using LAMP. The position of the SSR motif within the LAMP assay are shown. Coloured boxes denote the sequence at the 5' of the lamp primers that are present 3' of the priming sites required for hairpin formation. The * denotes the labelled primer that allows visualisation of the SSR-containing products. Corresponding PCR products were amplified using primers 1 and 3.

The SSR products from different rice lines amplified by LAMP and PCR are shown in Figure 2. The sizes of the products are consistent with the expected amplicons and two nucleotide differences between the varieties reflect the variation in copy numbers of the dinucleotide repeats. There is correspondence between the sizes of the SSR fragments produced by LAMP and PCR (Table 3). One variety 'Augusto' failed to amplify any products using LAMP (null allele) or when using the two displacement primers in PCR (data not shown). The failure in the two assays suggests that sequence polymorphism(s) around the SSR is preventing priming from some of the primers. This makes it highly improbable that 'Augusto Red' has been derived from a reversion of 'Augusto', as the name implies, since they differ not only in the pericarp colour but also in sequence around this particular SSR locus. Furthermore sequence data of the *Rc* gene for these two samples (not shown) show 'Augusto Red' has red pericarp because it has the wild-type *Rc* allele and not due to reversion of the 14 bp deletion, the predominant white rice allelic variant (Sweeney et al., 2007) found in 'Augusto' as demonstrated for true revertants of white rice (Brooks et al., 2008; Lee et al., 2009b).

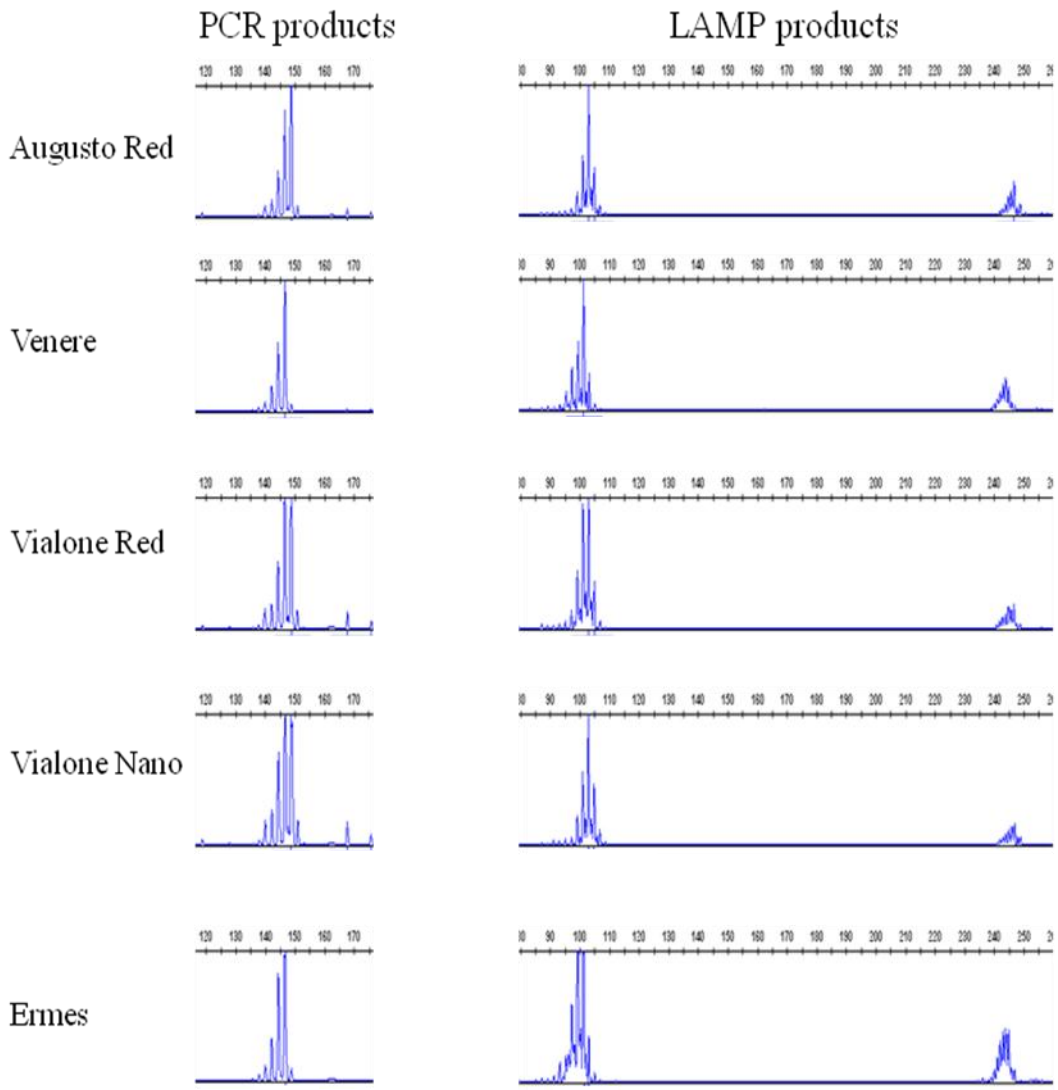

Figure 2. SSR products amplified using different assays. PCR (left) and LAMP (right) products visualised on an automated genotyper are shown. There is concordance between the different alleles between the assays. Secondary LAMP products (~240 nucleotides) are predicted from the inverted repeats produced by LAMP. Apparent sizes of the products (nucleotides) are shown above the peaks.

Rice sample	PCR (fragment size)	LAMP (fragment size)
Augusto	148	Null
Augusto Red	148	103/248*
Venere	146	101/244*
Vialone Red	148	103/248*
Vialone Nano	148	103/248*
Ermes	146	101/244*

null = no products; * denotes the secondary product size

Table 3. Amplification products from using PCR and LAMP assays. Sizes are given in nucleotides.

In LAMP reactions a secondary band, approximately twice the size of the smaller main band can be observed (Figure 2). Since LAMP produces a series of direct repeats that are composed of pseudo-inverted repeats, this secondary band corresponds to an inverted repeat amplified from

loop primer-loop primer product. The observation that allelic variants of this fragment differ by 4 nucleotides is consistent with there being two copies of the SSRs, in inverted orientation, within these fragments.

LAMP amplification has been shown to have near single copy target sensitivity (Lee et al., 2009c). To test the sensitivity of LAMP for the amplification of SSRs, a DNA sample from an inter-specific hybrid was used. The sensitivity of LAMP was demonstrated in two ways: dilutions of the DNA showed amplification at 10^{-4} dilution of a 20 ng sample. At this dilution, the amount of DNA in the reaction equates to 5 rice genomes. In previous studies quantifying DNA through single molecule amplification (Lee et al., 2010; La Mura et al., 2011) we know that DNA loss can considerable during *in vitro* manipulations if steps are not taken to minimise DNA adhesion to the plasticware. It was unlikely there were more than a couple of targets in the reaction. The verification that the low copy number of targets can be better visualised by the stochastic effects of amplifying from very low numbers of targets - the amplification of one allele from the heterozygote sample (Figure 3).

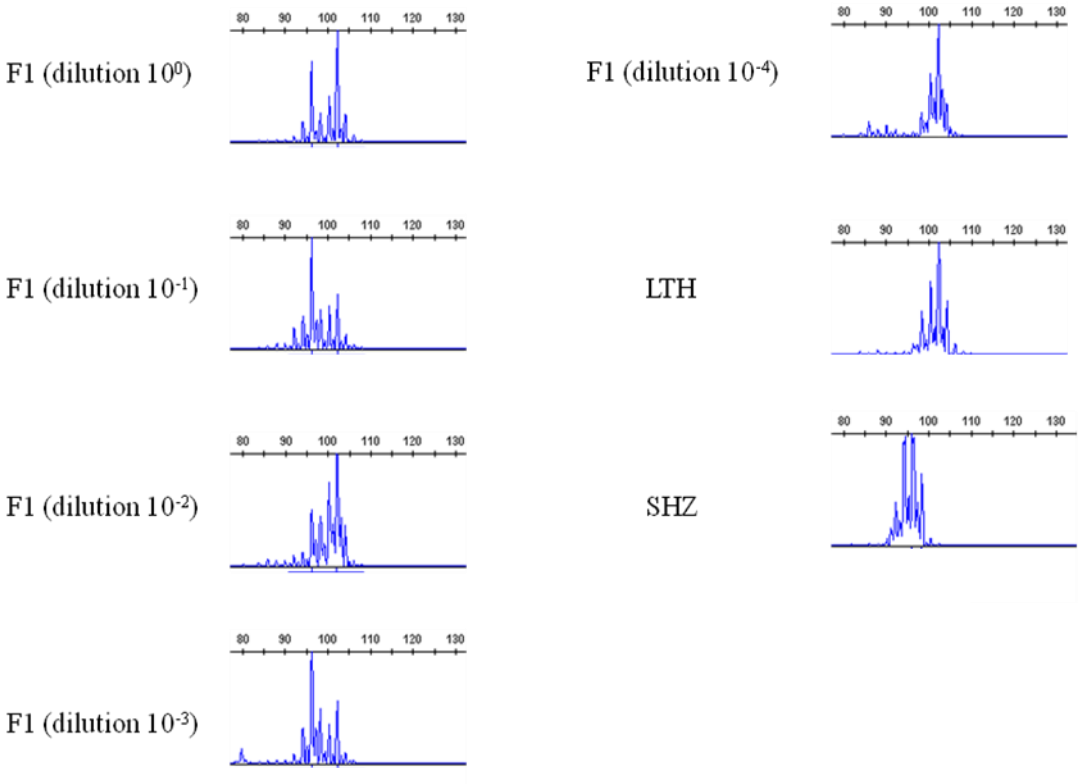

Figure 3. Amplification of rice SSR using LAMP. The SSR alleles of LTH and SHZ are shown together with the profiles of serial dilutions of an F1 sample. At low DNA concentrations (10^{-4} dilution), only one parental allele is amplified due to randomly picking up one allele in from the DNA sample.

SSR-containing fragments, amplified by PCR often exhibit the phenomenon of 'stuttering', where each SSR product can be seen as multiple bands. Multiple bands can be seen when SSR fragments are amplified using LAMP. Since LAMP is a continuous DNA copying process unlike PCR which is punctuated by denaturation, primer annealing and extension, the amplification of stutters by LAMP demonstrates stuttering is not unique to PCR. That multiple bands are observed even when amplifying from near single copies of the target argues against stutters reflecting the mosaic nature of plants (Cherfas, 1985), but that mutations that generate variation in SSR repeats are a natural consequence of replicating short tandem repeats.

These results presented demonstrate the use of LAMP to amplify SSR loci is extremely sensitive. Together with the known robustness of LAMP reactions, SSR genotyping by LAMP may find applications where sample is limiting and/or where a large number of samples need to be tested for small number of markers, for example, fine mapping to find recombinants across a small genetic region, allowing high throughput screening of SSR markers without DNA extraction.

Furthermore, the isothermal nature of LAMP makes it unlikely to undergo template-jumping that is observed in PCR of damaged DNA (Pääbo et al., 1990). Tandem repeating SSRs may be more prone to template jumping due to the reiterating sequence structure within the fragments that may result in new allelic variants produced by '*in vitro* recombination' (Ramos et al., 2008). Thus LAMP may be more suitable for the examination and testing of archaeological samples that have undergone much degradation of its DNA. At worst the samples will yield no products.

References

Akkinepalli, H., Ereful, N., Liu, Y., Malabanan, K., Howells, R., Stamati, K., Powell, W., Leung, H., Greenland, A., Mackay, I., Lee, D. (2012) Snapshots of gene expression in rice: limitations for allelic expression imbalance determination. Submitted to *Genome*.

Brooks, S.A., Yan, W., Jackson, A.K., Deren, C.W. (2008) A natural mutation in rc reverts white-rice-pericarp to red and results in a new, dominant, wild-type allele: Rc-g. *Theoretical and Applied Genetics*, **117**, 575-580.

Cherfas, J. (1985) When is a tree more than a tree? *New Scientist*, **20**, 42-45.

Fire, A. & Xu, S.Q. (1995) Rolling replication of short DNA circles. *Proceedings of the National Academy of Sciences of the United States of America*, **92,** 4641-4645.

Hosono, S., Faruqi, A.F., Dean, F.B., Du, Y., Sun, Z., Wu, X., Du, J., Kingsmore, S.F., Egholm, M., Lasken, R.S. (2003) Unbiased whole-genome amplification directly from clinical samples. *Genome Research*, **13**, 954-964.

Iwasaki, M., Yonekawa, T., Otsuka, K., Suzuki, W., Nagamine, K., Hase, T., Tatsumi, K., Horigome, T., Notomi, T., Kanda, H. (2003) Validation of the loop-mediated isothermal amplification method for single nucleotide polymorphism genotyping with whole blood. *Genome Letters*, **2**, 119-126.

Jones, H., Bernole, A., Jensen, L.B., Horsnell, R.H., Law, J.R., Cooke, R.J., Norris, C.E. (2011) Minimising inter-laboratory variation when constructing a molecular database of plant varieties in an allogamous crop. *Theoretical and Applied Genetics*, **117**, 1335-1344.

La Mura, M., Allnutt, T.R., Greenland, A., Mackay, I., Lee,D. (2011) Application of QUIZ for GM quantification in food. *Food Chemistry*, **125**, 1131-1520.

Lee, D., La Mura, M., Allnutt, T.R., Powell,W. (2009a) Detection of genetically modified organisms (GMOs) using isothermal amplification of target DNA sequences. *BMC Biotechnology*, **9**, 7.

Lee, D., Lupotto, E., Powell, W (2009b) G-string slippage turns white rice red. *Genome*, **52,** 490-493.

Lee, D., La Mura, M., Allnutt, T., Powell, W., Greenland, A. (2009c) Isothermal amplification of GM DNA sequences directly from plant tissues lowers the barriers to high-throughput and field-based genotyping. *Journal of Agricultural and Food Chemistry*, **57**, 9400-9402.

Lee, D., La Mura, M., Greenland, A., Mackay, I. (2010) Quantitation using informative zeros (QUIZ): application for GMO detection and quantification without recourse to certified reference material. *Food Chemistry*, **118**, 974-978.

Mitani, Y., Lezhava, A., Kawai, Y., Kikuchi, T., Oguchi-Katayama, A., Kogo, Y., Itoh, M., Miyagi, T., Takakura, H., Hoshi, K., Kato, C., Arakawa, T., Shibata, K., Fukui, K., Masui, R., Kuramitsu, S., Kiyotani, K., Chalk, A., Tsunekawa, K., Murakami, M., Kamataki, T., Oka, T., Shimada, H., Cizdziel, P.E., Hayashizaki, Y. (2007) Rapid SNP diagnostics using asymmetric isothermal amplification and a new mismatch-suppression technology. *Nature Methods*, **4**, 257-262.

Nagamine, K., Hase, T., Notomi, T. (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, **16**, 223-229.

Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B.P., Landegren, U. (1994) Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science*, **265**, 2085-2088.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, **28**, e63.

Pääbo, S., Irwin, D.M., Wilson, A.C. (1990) DNA damage promotes jumping between templates during enzymatic amplification. *The Journal of Biological Chemistry*, **265**, 4718-4721.

Ramos, M.D., Lalueza, C., Girbau, E., Pérez-Pérez, A., Quevedo, S., Turbón, D., Estivill, X. (2008) Amplifying dinucleotide microsatellite loci from bone and tooth samples of up to 5000 years of age: more inconsistency than usefulness. *Human genetics*, **96**, 205-212.

Saiki, R.K., Sharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim, N. (1985) Enzymic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, **230**, 1350-1354.

Sweeney, M.T., Thomson, M.J., Cho, Y.G., Park, Y.J., Williamson, S.H., Bustamante, C.D., McCouch, S.R. (2007) Global dissemination of a single mutation conferring white pericarp in rice. *PLoS Genetics*, **3**, e133.

Tomita, N., Mori, Y., Kanda, H., Notomi, T. (2008) Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature Protocols*, **3**, 877-882.

Vincent, M., Xu, Y., Kong, H. (2004) Helicase-dependent isothermal DNA amplification. *EMBO Reports*, **5**, 795-800.

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