To

My beloved husband Nuno

My wonderful and beloved sons Manuel José

José Pedro

Luís Filipe

Ana Carolina

and Tomás

Thank you for being my family the most precious thing in my life.

DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Lathyrus sativus L.

Application of Biotechnological and Biochemistry tecniques towards plant breeding

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Abbreviations

[^{14}C]- carbon14
^{1}H-NMR- proton nuclear magnetic resonance
AFLP- amplified fragment length polymorphism
AMOVA - analysis of molecular variance
AMPA- α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANFs- anti nutritional factors
ANOVA- analysis of variance
APS- ammonium persulfate
atm- atmosphere
ATP- adenosine triphosphate
BAP- benzylaminopurine
BAPN-β-aminopropionitrile
BC- before Christ
BIA- β-(isoxazolin-5on-2-yl)-alanine
BOAA - beta-oxalylamino-L-alanine acid
bp- base pair
Ca^{2+} - calcium ion
cDNA- complementary DNA
cm- centimeter
cm^3- cubic centimeter
CTAB- cetyltrimethylammonium bromide
CZE- capillary zone electrophoresis
D_2O- heavy water
DAP- 2,3-diaminopropionic acid
DAPA- oxalylation of 2,3-diamonopropionic acid
DNA- deoxyribonucleic acid
dNTP- deoxynucleotide triphosphates
DPSA- proportion-of-shared-alleles distance
EDTA- ethylenediamine tetraacetic acid
EST-SSR- expressed sequence tag – simple sequence repeat
FAO - Food and Agriculture Organization of the United Nations
FCA- factorial correspondence analysis
FDNB- 1-fluoro-2, 4 – dinitrobenzene
FIDs -Fourier transformation free-induction-decays
F_{IS}- inbreeding coefficient
FLB - formamide Loading Buffer
FMOC- 9-fluorenyl methylchloroformate

***F_{ST}*-** fixation index for population differentiation
g- gram
g/ mol- gram mol
g/L- gram per liter
GC-MS- gas chromatography-mass spectrometry
H_2O- water
ha- hectar
HCl- hydrochloric acid
***H_E*-** expected heterozygosity
***H_O*-** observed heterozygosity
HOD-residual solvent
HPLC- high performance liquid chromatography
hr- hour
IAA- indole-3-acetic acid
IBA- indole-3- butyric acid
ICARDA - International Center for Agricultural Research in the Dry Areas
IRD- infrared dye-labeled
kg- kilogram
kg/ha- kilogram per hectar
KOH- potassium chloride
Lat- latitude
Lon- longitude
LS1-8- *Lathyrus sativus* population nº 1 to 8
M13- M13 tailed primer
mA- milliampere
mg- milligram
mg/mL- milligram per milliliter
$MgCl_2$- magnesium chloride
min- minute
mL- milliliter
mm- millimeter
mM- millimolar
Mn^{2+}- manganese ion
MS- Murashige and Skoog nutrient medium (1962)
N- north
NAA- α–naphthalene acetic acid
N_{ar} - allelic richness
N_{av} **-** average number of alleles per locus
ng- nanogram
ng/ µL- nanogram per microlliter
NIR- near infrared reflectance spectroscopy
nº- number

N_{pr} - number of private alleles

NUCs- Neglected and Underutilized Crops

^{o}C- degrees Celsius

OPA- O-phthalaldehydo

PCR- polymerase chain reaction

PIC- polymorphism information content

ppm- parts per million

PVP- polyvinylpyrrolidone

r/s- rotations per second

rf -radiofrequency

RNAse- ribonuclease

rpm- rotation per minute

s- second

SE- standard error

SSR - Simple Sequence Repeats

T- temperature

t/ha- tons per hectar

TBE- tris/Borate/EDTA buffer

TEMED- tetramethylethylenediamine

TE–tris/EDTA buffer

UV- ultraviolet

V- volt

v/v- volume/volume

W- watts

W- west

w/v- weight per volume

β-ODAP- β-N-oxalyl- diamino-propionic acid

µL- microlliter

µM- micro molar

α-ODAP- α-N-oxalyl-diamino-propionic acid

Abstract

Grass pea (*Lathyrus sativus* L.) is one of the pulse underutilized crops (NUC) that can provide an important source of rich-protein food, mainly in low-income food deficit countries, since it can thrive in marginal lands where modern crops are unable to grow. Moreover, *L. sativus* may be an important source of genetic diversity, with very interesting traits, which will be very useful for plant breeding programs. Despite the many advantages that grass pea presents, the full potential of *L. sativus* has not been realized. In recent years these has been a renewed interest on this crop, Portugal included, and some of the most recent biotechniques have been used to address the improvement of grass pea.

The purpose of this work is to make a study of eight *L. sativus* Portuguese landraces addressing three different, yet complementary aspects of grass pea improvement: propagation through tissue culture; evaluation of genetic diversity and identification and quantification of the neurotoxin β- ODAP.

Seeds of eight different genotypes of *L. sativus* landraces were collected from eight farms from Sicó region. Shoot tip cultures were established *in vitro* on a MS medium supplemented with 1 µM BAP. No differences were found in the response of the different genotypes. In a second experiment shoot tips from the best responding genotype (LS2) were tested on media containing different concentrations of BAP (0, 1, 2, 4, 8 and 10 µM) with 2 and 10 µM of this cytokinin giving the best results (5.02 cm average length of shoots in 2 µM and 1.7 average shoot per explants in 10 µM). Attempts to root the obtained shoots on a medium containing 2.46 µM IBA followed by transfer to an auxin-free medium only had little succeed with only a few shoots showing root formation.

Data of the genetic diversity study indicate that no significant differences between the two collecting sample areas (Penela and Alvaiázere) were found. The high genetic diversity was on average 0.438, being the highest value (0.515) detected on LS7 population and the lowest (0.270) on LS4 population. Considering the result of the AMOVA most of the molecular variance detected is due to differences within

populations (93.27 %) and only 6.73 % of the variance can be associated to differences among populations.

A proton (^1H) Nuclear Magnetic Resonance (NMR) spectroscopy was performed to identify and quantify the β- ODAP content among the different eight populations. The results of ^1H-NMR analysis showed that the β-ODAP content is similar among the populations, ranging from 0.366-0.572 ppm. LS3 possesses the lowest content (0.366 ppm) while LS4 possesses the highest content (0.572). The results demonstrate that β-ODAP is the only isomer present in the samples of *L. sativus*. The conversion of this isomer into the α-ODAP requires thermal activation indeed boiling of the seed is able to reduce the levels of β-ODAP to almost 56% after heating for 60 minutes.

Key Words: EST-SSR, genetic diversity, *Lathyrus sativus* L., micropropagation, β-ODAP.

Resumo

O chícharo (*Lathyrus sativus* L.) é uma das culturas de grão que tem sido negligenciada (NUC), no entanto apresenta um elevado potencial para ser uma importante fonte de proteínas, principalmente em países com deficit alimentar, uma vez que consegue desenvolver-se em terrenos marginais inapropriados para outras culturas mais exigentes. Ademais, o chícharo pode ser um importante reservatório de diversidade genética, com características de elevado interesse, que poderá ser utilizado em programas de melhoramento vegetal. Apesar das enormes vantagens que o chícharo comporta, todo o seu potencial está ainda por realizar. Recentemente tem havido um renovado interesse nesta cultura, inclusive em Portugal, e algumas das mais modernas biotecnologias têm sido aplicadas no sentido de promover o melhoramento desta planta.

O objetivo deste trabalho é a realização do um estudo de oito variedades tradicionais portuguesas abrangendo três diferentes, mas complementares, aspetos concomitantes com o melhoramento do chícharo: propagação através da cultura de tecidos, avaliação da diversidade genética e identificação e quantificação da neurotoxina β- ODAP.

Colheram-se, junto dos produtores da região do Sicó, oito genótipos das variedades tradicionais. Foram estabelecidas culturas *in vitro* de ápices meristemáticos em meio MS suplementado com 1 μM BAP, não tendo sido observadas diferenças relevantes na resposta dos diferentes genótipos, contudo o genótipo LS2 foi o que apresentou melhores resultados. Numa segunda experiência foram testados explantes do genótipo (LS2) em meio de cultura com diferentes concentrações de BAP (0, 1, 2, 4, 8 e 10 μM), tendo sidos as concentrações de 2 e 10 μM a apresentar os melhores resultados (5.02 cm comprimento médio dos rebentos para 2 μM e 1,7 média de rebentos por explante para 10 μM). No que concerne ao enraizamento, explantes colocados em meio contendo 2,46 μM IBA e posteriormente transferidos para meio sem hormona, apenas alguns rebentos apresentaram a formação de raízes.

Os dados resultantes do estudo de diversidade indicam que não existem diferenças geneticamente relevantes entre as variedades colhidas em Alvaiázere e Penela. A elevada diversidade genética apresentou um valor médio de 0,438, com o

genótipo LS7 a refletir o valor mais alto (0,515) e o valor mais baixo (0,270) é atribuído ao genótipo LS4. Considerando os valores resultantes da AMOVA, a variância molecular é justificada pela diversidade intra variedades, entre os indivíduos (93,27%) e apenas 6,73% é atribuída às diferenças entre variedades.

Foi utilizada a tecnologia de Ressonância Magnética Nuclear (NMR) para identificar e quantificar os níveis de β- ODAP existentes nas diferentes variedades. Os resultados da NMR mostram a quantidade de β- ODAP é similar nas oito variedades, variando num intervalo 0,366-0,572 ppm. LS3 possui o valor mais baixo (0,366) enquanto que o genótipo LS4 apresenta o mais elevado (0,572). Os resultados demonstraram que o β-ODAP é o único isómero presente nas amostras analisadas. A conversão deste isómero em α-ODAP requer ativação térmica de facto, submeter o grão de chícharo a uma temperatura de 100ºC durante 60 minutos pode reduzir os teores de β-ODAP em cerca de 56%.

Palavras-chave: Diversidade genética, EST-SSR, *Lathyrus sativus* L., micropropagação, β-ODAP.

1. INTRODUCTION

1.1-Framework

The food crisis of 2008 clearly showed how far we are from achieving genuine food security. This crisis was a direct consequence of reduced stocks due to several years of low yields in major crops. Extreme weather events associated with the global economic downturn and with the increasing use of land for bio-fuel production may be signaled as the main events causing the world concern of 2008. Moreover, according to FAO projections, the world population will increase from the actual 7 billion to about 10 billion by 2050. If this scenario becomes true, food production in 2050 must increase by about 70% when compared to 2005 to maintain the levels of food security occurring in 2005 (FAO, 2010).

Nowadays, more than 1.5 billion hectares of the globe´s land surface (about 12%) are used for crop production. However, since the second half of the last century, this area has been consistently reduced and it does not seem likely an increase in the area of cultured crops in the next decades (Oldeman, 1992). In fact, and considering the available information, the contrary seems more plausible due to several factors such as soil erosion, salinization and nutrient exhaustion (FAO, 2010).

Despite these problems, food production has been kept in pace with the increasing of human population due mainly to improved technology, development of new crop varieties and the increase in efficiency of irrigation systems.

To maintain the levels of food production in the near future it will be necessary to continue with these progresses but, no less important, is to bring to the food production sector new species or cultivars that can be able to grow in more adverse weather and soil conditions. It is estimated that only 7.000 of the calculated 350.000 plants have been cultivated since the beginning of farming: however, only 30 crops provide around 90% of the world population dietary energy requirements (FAO, 2010). Reliance on a reduced number of crops can lead to rapid genetic erosion, increasing risks of new plant diseases and susceptibility to climate changes, resulting in food insecurity (FAO, 2010). Agricultural biodiversity is the result of both human and

natural selection. The diversity among crops is important to enabling balanced and nutritious diet and allows adapt food production to changing demands of the society.

Most of the crops that have potential to be cultured and used for food production, but which have not been properly explored are usually known as NUCs (non cultured or underutilized crops). These are species important for the agro-forestry sector but which, for any reason, economic, cultural or other are not extensively cultured. Most of these species have suffered reduced plant breeding and are able to thrive in marginal lands where modern crops are unable to grow. Moreover, some of this NUC species are genetically related with major crops, meaning that some of their interesting characteristics can be transferred to related varieties by sexual hybridization. Thus, NUCs are not only species that can be used as new sources of food or other commodities but are also an important source of genetic diversity which will be very useful for plant breeding programs (Gomes, 2011; Mal, 2007; Padulosi *et al.*, 1999).

One species which can be considered a NUC is *L. sativus*, a member of the Fabaceae family which is cultured in some regions of Portugal and around the world. This and other species must be better characterized in order to improve their quality and to bring them to the productivity sector, hence increasing the variability of the crops used in agriculture and contributing to a reduced dependence on a small number of genetic resources. Moreover, most of these species are poorly known both in terms of their genetic diversity but also in what concerns their biology. In this context, it is of almost importance to develop breeding programs for these species and to apply biotechnological tools to improve the characteristics more related with plant production.

1.2- The genus *Lathyrus* and the specie *Lathyrus sativus* L.

1.2.1- Characterization and taxonomy

L. sativus is one of the annual pulse crops belonging to the family Fabaceae and the tribe Fabeae (which is known as Vicieae). Fabaceae is the third largest family of flowering plants after the Asteraceae and Orchidaceae. The Fabaceae are notably 'generalists' ranging from forest giants to tiny ephemerals, with great diversity in their methods of acquiring the nutrients for development, reproduction and defense (Polhill *et*

al., 1981). The family can be found in all terrestrial habitats, from the equator to the polar fringes. However much of its diversity is centered in areas of varied topography and temperate climates (i.e. in the case of *Lathyrus* the Mediterranean basin) (Shehadeh, 2011).

There are about 150 species in the genus *Lathyrus* that comprises 15 sections (Smartt, 1994), distributed throughout temperate regions of the northern hemisphere and extends into tropical East Africa and into South America (Shehadeh, 2011).

Kupicha (1983) described the genus *Lathyrus* as perennial and annual herbs, with erect or more usually climbing or sprawling habit; rootstock occasionally tuberous. Stems winged or un-winged, always with complete replacement of cortical vascular bundles at the nodes. Leaves hypostomatic to epi-amphistomic, paripinnate ending in tendril or mucro; leaflets 1-8-paired (frequently 1-paired), entire, with supervolute venation and brochidodromous, veins pinnate or parallel. Leaves occasionally phyllodic or reduced to stipules and a tendril. Stipules entire and rarely toothed; semisagittate or hastate. Inflorescence racemose, 1-many-flowered. Calyx usually actinomorphic, sometimes with oblique mouth and teeth of unequal length. Standard oblong to stenonychioid usually bossed or pouched at the fold. Staminal tube usually truncates at apex, rarely oblique. Style dorsally compressed, pubescent on adaxial face, sometimes spathulate and/or contorted; stigma sometimes double. Legume compressed, sessile, rarely stipitate, sometimes winged, occasionally bearing glandular or tuberculate hairs, rarely villous, rarely with membranous or woolly partitions between the seeds, legume 2-many-seeded. Seeds with long to short hilum; testa smooth or rough; lens always near hilum; free amino acid canavanine absent, lathyrine often present. x=7, polyploidy rare. Figure 1 shows some details of *L. sativus*.

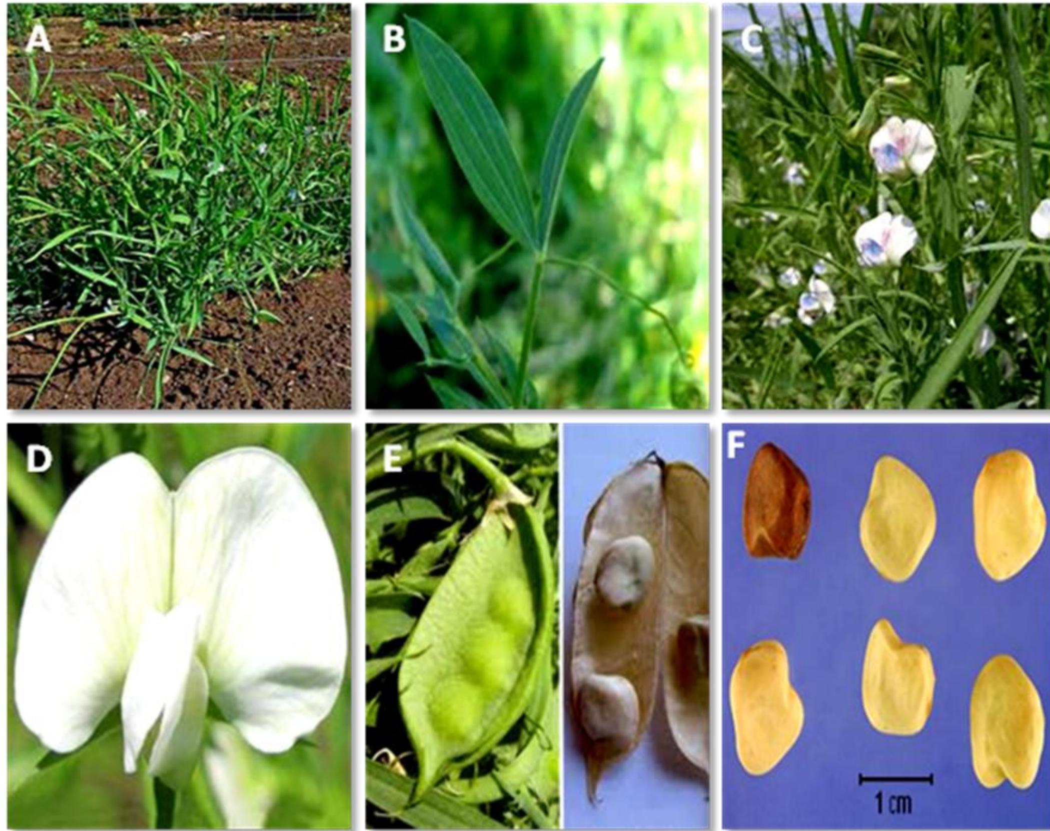

Figure 1 – Several aspects of *L. sativus*. (A)- *L. sativus* plant. (B)-A paired leaflet. (C)- *L.sativus* plant with blue-pink flowers. (D)- Detail of a white flower. (E)- Pods in different stage of maturity. (F)- Seeds of *L.sativus*: white and larger seeds typical from the Mediterranean and more dark seeds typically from the west European region (from- http://plants.usda.gov).

Although the genus *Lathyrus* comprises a great number of annual and perennial autogamous and allogamous herbaceous creeping plants (Kupicha, 1983), only a few species are widely cultivated as food or fodder crops (Vaz Patto *et al*., 2006a). The economically important species cultivated are *L. cicera* (chickling vetch), *L. tingitanus* and, especially, *L. sativus* (grass pea). Others, such as *L. ochrus, L. latifolius* and *L. sylvestris* are important forage crops. The sweet pea (*L. odoratus*) is valued as ornamental specie (Campbell, 1997).

Grass pea an annual grain legume is also known as *chícharo* in Portugal, *almorta or alverjón* in Spain, *cicerchia* in Italy, *guaya* in Ethiopia, *khesari* in Bangladesh, *pois carré* in France, *san lee dow* in China and *khesari dal* or *theora* in India (Kumar *et al*., 2011).

L. sativus is predominantly a self-polinated crop, although a significant out-crossing rate has been reported: 4-16% according to Kaul *et al*. (1986), 28% in Bangladesh (Rahman *et al*., 1995) and Gutierrez-Marcos *et al*. (2006) calculated out-crossing to be 36%. Entomophilic pollination in grass pea is due mainly to bees. Flower color and size have shown their influence on the out-crossing rate. The highest out-crossing (27.8%) has been reported in varieties with red flowers followed by pink (19.4%) and white (9.8%) in Bangladesh (Rahman *et al*., 1995). Large size of flowers, bright color of petals, flower density and nectar production are reported to influence the out-crossing in *Lathyrus* species (Kiyoshi *et al.*, 1985). In the Mediterranean environment, 20-27% out-crossing has been observed depending on the local environmental conditions (Kumar *et al.*, 2011).

1.2.2- Origin and distribution

The origin of grass pea is not completely clarified however, according to Smartt (1994) one of the centers of origin is south-west or central Asia. Vavilov (1951) proposed two distinct centers of origin for *Lathyrus*, one in central Asia, including India, Afghanistan, Republics of Tajikistan and Uzbekistan, and the other in the center of Abyssinia. All grass pea lines appear to be divided into two geographical origins – one group derives from the Indian subcontinent, and another from the Mediterranean/ European region, which typically has higher yields and larger seeds (Hanbury *et al*., 1999). Some diversity studies performed with grass pea populations revealed two groups of *L. sativus* accessions: white-seeded with large seeds, originating mainly from Europe and north Africa, and colored-seeded with relatively small seeds, originated mainly from Asia and Ethiopia (Przybylska *et al.*, 2000). Yunus & Jackson (1991), based on the size of the vegetative organs, flower and seed color observed a clear division into two groups. One of the genotypes consisted in blue flowers and is originated from southeast and south of Asia and Ethiopia, and another one with white and white/blue flowers has its origin in Mediterranean region.

After combining archaeobotanical and phylogeographical evidences, it has been found that the cultivation of grass pea began in the Balkan Peninsula, during the early Neolithic period, near the beginning of the 6th millennium BC (Kislev, 1989), and later on diffused through Europe and Mediterranean regions. Charred seeds of *Lathyrus* sp.

have been found in Israel and it is believed that seeds were carried to the Levant from the Aegean, in the Bronze Age (Mahler-Slasky & Kislev, 2010). Carbonized *Lathyrus* seeds have been retrieved from a number of pre-historic sites in Greece and Iran and could be also traced in Italy and Southeast France. One isolated sample is reported from early Bronze Age in Portugal and the most Northern findings were reported in Hungary (Campbell, 1997).

Simola (1968), as resumed by Mahler-Slasky & Kislev (2010a), suggested South America origin of *Lathyrus* followed by a dispersal event to Africa and then into the Mediterranean region. In contrast, Kupricha (1983) suggested that *Lathyrus* originated at high latitudes in the Old World and might have migrated later to the Mediterranean region and to North America via Greenland or from Asia via Beringia to Alaska. From North America, the lineages could have spread into South America in the late Tertiary, as resumed by Schaefer *et al.* (2012).

Based on DNA sequence data, Kenicer *et al.* (2005) suggested an eastern Mediterranean origin for *Lathyrus* followed by range expansion into northern Eurasia. The Beringian land bridge would then have allowed migration into North America. According to Kenicer *et al.* (2005), the most likely origin of the South American *Lathyrus* species is directly from Eurasia.

1.2.3 – Grass pea features and uses

Grass pea is one of the few crops that can provide the bulk of food consumer in the world. Several *Lathyrus* species and mainly *L. sativus* have great agronomic potential and represent an alternative pulse in many cropping systems around the world (Siddique *et al.*, 1996).

Grass pea perform well under adverse agricultural conditions, and its many cultivars possess different attributes such as the ability to resist to drought and flooding, high climatic adaptability and the ability to grow in cool climates and at high altitudes (McCutchan, 2003). It is also important the capacity of this crop to adapt to salinity, alkaline, clay or otherwise poor soils due to its hardiness (Berger *et al.*, 1999; Chen *et al.*, 2000). Root nodules of this species are able to fix atmospheric nitrogen which makes it interesting for rotation with other crops, in particular cereals. Reports have estimated an increase of 67 kg/ha of nitrogen in a single season (McCutchan, 2003).

Including grass pea in crop rotations, mainly with wheat, can bring several benefits specially a better disease control, nutrient recycling, improvement of the soil structure and protection against the erosion (Chatel *et al.*,1982). According to Abd El-Moneim *et al.* (1993) the introduction of grass pea in a crop rotation system can turn the production system more sustainable due to the soil fertility improvement and diseases and pests cycle breaking making this species particularly suitable for integrated management agriculture systems.

It is considered one of the species that can be an important alternative source of protein (27-29%) for human consumption and animal feeding in addition to an excellent flavor (Hillocks & Maruthi, 2012).

There is a great potential for the expansion in the utilization of grass pea in dry areas and zones which are becoming more drought-prone, and where the prices of food are rising as a result of natural disasters associated with climate changes (Hillocks & Maruthi, 2012). Due to expected climate changes, there are serious concerns regarding the sustainability of agricultural production and food security. Species more amenable to growth in these changing environments, as *L. sativus*, can play an important role in the near future (Kumar, 2011).

In studies performed in hydroponic cultures, *L. sativus* revealed a high tolerance to lead, which was retained in the root system. There was also a decrease of calcium, zinc and copper in root tissues suggesting a high tolerance to the deficiency of essential nutrients. Given these findings, *L. sativus* presents itself as an interesting candidate species in bioremediation (Brunet *et al*., 2008).

To Smartt (1994) is quite surprising that a culture so spread and cultivated around the world has evolved so little. This author believes that, probably, the lack of evolution as a grain legume is due to the pressure imposed on the evolution as forage crop. Getting more and large seeds is advantageous for grain crop, but is not relevant in a forage species. To Vaz Patto *et al*. (2011), one important reason for the little research and development of these specie is the lack of governmental funding and interest of the private sector.

According to (Hillocks & Maruthi, 2012) there is sufficient variability in numerous germplasm collections of *L. sativus* around the world large knowledge and techniques to provide a platform for the development of grass pea as a model crop for sustainable farming systems in harsh environments and for climate change adaptation.

1.2.4- Economic interest and agronomy

Statistics about grass pea, both in terms of production and yield are not readily accessible. However, according to Skiba *et al*. (2007) India is the largest producer of grass pea grain, representing an acreage, in 1995, of 1.4 million ha and an average yield of 0.4-0.6 t/ha (Gautam, *et al.*,1998). According to Pandey *et al*. (1996) in India, grass pea is cultivated under three farming systems:

- As a sole crop in rainfed areas where the irrigation is not available for winter crop and soil are vertisoils.
- Relay or “utera” systems where the grass pea seeds are broadcasted in standing crop of rice, nearly 20-30 days before the harvest of rice
- Mixed cropping, in rainfed areas where grass pea is grown as mixed crop with wheat, chickpea, barley and linseed.

Bangladesh and Ethiopia are, respectively, the second and third largest producers with 0.24 million ha of cultivated area and an average yield 0.7 t/ha in Bangladesh (Sarwar *et al*., 1996) where grass pea in the most important pulse crop (Kuo *et al*., 2000). In Bangladesh, grass pea is grown mostly in low lying areas among rice fields. It is also grown smaller areas as an intercrop in consociation with sugarcane. Some rhizobial strains compatible with grass pea nodule formation for high yield have been identified. Therefore chemical fertilizers are not used in its cultivation (Malek *et al*., 1999).

In Ethiopia the average yield is 0.7 t/ha being the cultivated area of 0.14 million ha (Tadesse & Bekele, 2003b). During the severe droughts that often occur in this country, the grass pea is usually the only plant that can continue to produce where all others fail, thus ensuring the survival in affected areas (Fikre *et al*., 2008).

L. sativus is also cultivated less extensively in other several countries such as Nepal and Pakistan (Neupane, 1996). In this last country the pulse crops accounts for 7-8% of the total cropped area of the country. Among these, grass pea is the second most cultured, after *Cicer arietinum* (Haqqani & Arshad, 1996). In Pakistan, a rice-grass pea-rice is the most common rotation.

In all these countries, grass pea is recognized as a food of exceptional value, in particular among the poor farming communities because of its lower prices, high protein levels and the ability to grow in poor soils. However, the culture of *L. sativus* is not limited to these countries. According to Campbell (1997), Southern and Western Europe, West Asia and North Africa are some of other areas were grass pea is cultivated. According to Vaz Patto *et al.* (2006b), in Europe, where diversification of cropping systems and more sustainable and environmentally safe agriculture are main concerns, grass pea has the potential to be reintroduced in marginal areas providing an alternative to imported soya or to the hazardous use of protein of animal origin in animal feeding.

In zones of Australia showing Mediterranean climate type, grass pea has been introduced as a grain legume to be include in cropping rotations with wheat, in soils unsuitable for the production of others grain legume such as lupin (*Lupinus angustifolius*), pea (*Pisum sativum*) chickpea (*Cicer arietinum*), etc. Another important grass pea feature for its introduction in Australia, is the ability for resistance disease (Siddique *et al.*1996; Hanbury *et al.*, 1999), to enriching soil culture as a break crop between cereals and as a bonus crop in fallow land, mainly in the regions with less than 300 mm of annual precipitation (Kumar *et al.*, 2011).

In Poland there has been recent interest in selection of improved grass pea lines due to their suitability for adaptation to climate changes and sustainable farming systems on poor soils (Grela *et al.*, 2012). Also in Italy there is a renewed interest on this crop because in can provide an efficient alternative for areas where land is over exploited by excessive cereal cultivation (Tavoletti *et al.*, 2005). Table I shows the current importance of *L. sativus* world cultivation.

In Portugal, statistical data about *L. sativus* are not available. However, there is an increasing interest on this NUC, special in the mountainous region of Serra do Sicó,

were grass pea has been cultured for a long time. The problem is that the cultures are of small scale and the production is mainly used for self consumption rather than for commercial production (Vaz Patto, 2009). Tanks to the empowerment of the local authorities in supporting and organizing an annual initiative (Festival do Chícharo), the grass pea has became progressively more well-known in our country. This and the economic crisis have driven the attention of young farmers who are now quite interested in the culture and production of species which have been used for many years in our agriculture but which are now well known in global markets.

The information about the culture of *L. sativus* in Portugal is scarce. However, in the Alvaiázere region, farmers cultivate grass pea in a crop rotation system, mainly alternating with cereals; in intercropping with olive (*Olea europea*), chickpea (*Cicer arietinum*), broad bean (*Vicia faba*) or as an isolated crop for seed production. There are no official records about the yield of this crop, yet supported on the information coming from the farmers, the yield in Sicó region is around 0.8 t/ha. Nevertheless under favorable climatic conditions may yield over than 3 t/ha of seed (Krarup, 2002; Mera *et al.*, 2003b).

Table I- Current importance of *L. sativus* world cultivation

Countries where *L.sativus* is currently an important crop	
Bangladesh	Campbell *et al.* (1994); Rahman *et al.* (2001)
China	Campbell *et al.* (1994); Zhou and Arora(1995)
Ethiopia	Campbell (1997); Tadesse and Bekele (2003a)
India	Campbell *et al.* (1994); Pandey *et al.* (1995), Sarkar *et al.* (2003)
Nepal	Campbell *et al.* (1994); Neupane (1995)
Pakistan	Campbell *et al.* (1994); Haqqani and Arshad (1995)
Countries where *L.sativus* is grown to a lesser extent	
Australia	Hanbury *et al.* (1999)
Europe (Italy, France, Poland, Portugal)	Campbell *et al.* (1994); Tavoletti and Capitani (2000); De la Rosa and Martin (2001), Milezak *et al.* (2001)
North Africa (Egypt, Morocco, Algeria)	Campbell (1997)
South America (Chile, Brazil…)	Campbell *et al.* (1994); Mera *et al.* (2000)
West Asia (Syria, Lebanon, Iraq, Afghanistan...)	Campbell *et al.* (1994); Campbell (1997)

Based on Vaz Patto *et al.* (2006)

1.3- Nutritional value of *Lathyrus sativus* L.

The nutritional value of winter crop grass pea is determined by the amount of biologically available nutrients and the effects of their anti nutrients (Enneking, 2011).

1.3.1- Available nutrients and ANFs

The composition in the seed of *L.cicera* and *L.sativus* is similar to that of other grain legumes with high amounts of protein. The amount of protein present in dry grass pea seeds is between 21 and 25%. Gatta *et al.* (2002) studying 161 lines of *L.sativus* reported that the protein content in the seeds ranges from 23% to 29.9%.

These proteins are rich in amino acids such aslysine, but usually poor in sulfur-rich methionine and cysteine aminoacids (Fikre *et al.*, 2008). These researchers also found that, in different genotypes, glutamic acid is usually present at higher concentrations, followed by aspartic acid, arginine and lysine. A similar pattern was found for soybeans and lentils, except for the leucine which showed that a higher concentration than lysine (Fikre *et al.*, 2008). The sulfur amino acid methionine showed lower values for all genotypes of *L. sativus,* similar to lentils but only half of soybean, grain legume known as one of the richest sources of protein.

Chemical analysis showed that the seeds of *L.sativus* and *L.cicera* contain similar levels of protein, fat, starch, minerals, metabolizable energy than those of peas *(Pisum sativum*) and broad beans *(Vicia faba*). Moreover, digestibility is also similar to those species. Methionine and cysteine are present in low levels whereas lysine and phytate are present in high amounts (Enneking, 2011).

Grass pea contains high levels of anti nutritional factors (ANFs, Wang *et al.*, 1998) especially the neurotoxin β-ODAP (β-N-oxalyl-L-α, β-diaminopropionic acid). In 1964, β-ODAP was considered the cause of neurolathyrism (Murti *et al.* 1964, Rao *et al.*, 1964). In *L. cicera* β-ODAP levels are lower (about 0.16%) while in *L. sativus* values are about three times higher (Enneking, 2011). Hanbury *et al.* (2000) reported that the average concentration of β-ODAP, for *L. sativus* varies between 0.04 - 0.76% while *L. cicera* values ranges from 0.08 to 0.34%. The amounts of methionine appear to remain stable in different genotypes of *L. sativus,* even when comparing genotypes with a high amount of β-ODAP with genotypes that have low values of this compound. Nunn *et al.* (2005), reported that consumption of these seeds, limited in methionine, for extended periods can lead to deprivation of methionine in the blood plasma. Studies carried out in mice found that methionine is exclusively concentrated in neurons (Nunn *et al.*, 2005). If this happens to humans, prolonged lack of methionine in the plasma reduces the amount that enters in neuronal cells and may lead to increased susceptibility of these cells to the effects of excitatory β-ODAP.

The lipid content in the seeds of *L.sativus* is low (<2%) (Buchanan, 1994 as cited by Enneking, 2011); however, fatty acids present have a high nutritional value with higher proportions of palmitic acid and linoleic acids and minor amounts of oleic acid and arachidonic (Enneking, 2011).

1.3.2- β-ODAP – *L. sativus* neurotoxin

β-*N*-oxalyl-L-α,β-diaminopropionic acid, also known as ODAP, BOAA and β-oxalylamino-L-alanine, has a mass molar of 176.13 g/ mol and a chemical structure as illustrated in figure 2.

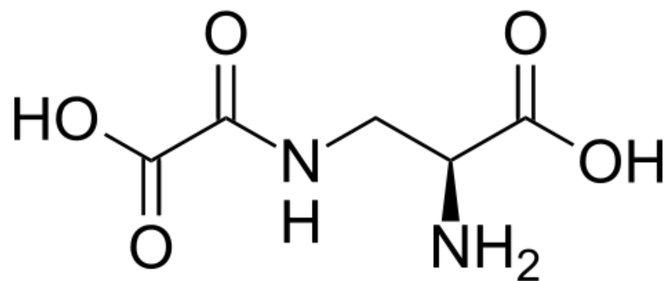

Figure 2- Chemical structure of β-*N*-oxalyl-L-α,β-diaminopropionic acid ($C_5H_8N_2O_5$)

While twenty amino acids are involved directly in protein structure, there are many of others, the non-protein amino acids, that possess different rules including antiherbivory, antimicrobial and allelochemical activity, protection against stress, signaling, nitrogen storage and as toxins against invertebrates and vertebrates (Vranova *et al.*, 2011).

The β-ODAP content in *L. sativus* depends of the genotype and environmental conditions (Campbell, 1997). Hanbury *et al.* (2000) evaluated the β-ODAP content of 407 *L. sativus* lines from three different locations in Australia, and 96 of *L. cicera* lines collected in Ethiopia, the Mediterranean and Europe, and concluded that the levels of β-ODAP in both species, are rather correlated with the genotype than to environmental conditions. However, Wuletaw (2003) who studied the stability of the levels of β-ODAP in *L.sativus,* showed a strong interaction between genotype and environmental conditions.

Species of *Lathyrus* differ in the content of ODAP in the seeds. Some, like *L. tingitamus*, do not have ODAP in their tissues while *L.sativus* and 20 other species accumulate varying amounts of the neurotoxin in there seeds (Addis & Narayan, 1994).

Variation in the content of ODAP was also reported among cultivars of *L.sativus* (Kumar *et al*., 2010), as can be seen in table II.

Table II- Genetic variation for ODAP content in grass pea germplasm

Country/ Institution	Number of accessions	ODAP (%) in seeds Minimum	Maximum	References
Bangladesh	172	0.450	1.400	Kaul *et al.*(1986)
Bangladesh	116	0.040	0.780	Sarwar *et al.(*1996)
China	73	0.075	0.993	Campbell *et al.(*1994)
Ethiopia	150	0.149	0.916	Tadesse & Bekele (2003)
India	576	0.100	2.590	Nagarajan & Gopalan (1968)
India	1500	0.150	0.300	Jeswani *et al. (1970*)
India	643	0.100	0.780	Somayajulu *et al. (1975*)
India	1000	0.200	2.000	Leakey (1979)
India	1187	0.128	0.872	Pandey *et al*. (1995, 1996, 1997a)
India	1963	0.067	0.712	Pandey *et al*. (2008)
ICARDA	81	0.020	0.740	Robertson & El-Moneim(1997)
ICARDA	1128	0.150	0.952	Personal communication (2009)
Australia	503	0.040	0.760	Hanbury *et al*.(1999)
Chile	76	0.180	0.520	Tay *et al*.(1999)

Based on Kumar *et al*. (2011)

The biosynthesis of secondary metabolite β-ODAP was proposed as an oxalylation of 2,3-diamonopropionic acid (DAPA) with oxalyl-coenzyme A, but the natural occurrence DAPA was never demonstrated. Further studies showed that DAPA is only a short-living intermediate between β-(isoxazolin-5on-2-yl)-alanine (BIA) and β-ODAP (Kuo *et al*., 1998). More detailed analyses using [^{14}C] confirmed that BIA is the precursor for ODAP and indicated that DAPA may indeed be the short-lived intermediated (Lambein *et al*., 1990).

The availability of many nutrients such as copper, magnesium, boron, molybdenum, cobalt, aluminum, zinc and iron and heavy metals (cadmium) has effects on β-ODAP biosynthesis. Haque *et al.* (2011) varied the concentrations of some

elements and found that the biosynthesis of β-ODAP from BIA in callus of *L.sativus*, increased in the presence of an excess or shortage of Mn^{2+}.

ODAP remains a useful neuro-active molecule that has been useful in the elucidation of important molecular mechanisms related with neurodegenerative diseases (Vaz Patto *et al*., 2011). It was shown that ODAP affect various neurological and enzymatic activities being essential to develop an animal model of neurolathyrism (Kusama-Eguchi *et al*., 2005) that can provide a better elucidation of more general molecular mechanisms of neurodegeneration.

Chromatography and chemical analysis have confirmed that the rate of biosynthesis and accumulation of β-ODAP in tissue changes considerably during ontogeny. The maximum accumulation occurs in two phases: during the reproductive period in which there is an increase in the rate of synthesis and accumulation in the epidermis of the pods, embryos, and in roots, and during the initial phase of vegetative development, with an increase in the amounts present in both stem and root apical meristems. During the intermediate stage of plant development very little ODAP has been detected (Addis & Narayan, 1994).

The strong similarity of the dissolved β-ODAP molecule to glutamate may explain the inhibition of glutamate transport into neuronal astrocyte cells. β-ODAP also exerts some toxicity against those cells that produce the energy-rich ATP for the neurons. This excitatory (β-ODAP) amino acid receptor binds acid, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) leading to degeneration of neurons, through the induction of cell death by excitotoxicity and increasing of oxidative stress(Moorhem, *et al*., 2011).

This toxin is known to disrupt mitochondrial respiratory chain, and recent data indicate that β-ODAP can inhibit the uptake of cysteine, compromising thus the cell's ability to deal with oxidative stress. It has been also suggested that β-ODAP disturbs the homeostasis of Ca^{2+} ion by increasing the amount of the mitochondrial endoplasmic reticulum (Moorhem *et al*., 2011). The signal transduction cascade has some aspects in common with other neurodegenerative diseases. Based on this mechanism, researchers (Moorhem *et al*., 2011) concluded that a diet supplement with methionine and cysteine can significantly reduce the risk of neurolathyrism.

Consumption of onion, ginger and garlic can have a protective effect against ODAP toxicity and when the consumption of grass pea is combined with cereals rich in sulphur amino acids, neurolathyrism is rarely a problem (Lambein *et al.*, 2007).

1.3.2.1- Isomerisation of β- ODAP

Naturally occurring ODAP exists in two isomeric forms, α and β, where α-form isomer is less toxic, (or even non-toxic) than the β-isomer form (Yigzaw *et al.*, 2001). Usually concentration of the β form is about 95% of the total ODAP (Kumar *et al.*, 2011). The isomerisation of β- to α-ODAP is time and temperature conditions dependent (Padmajaprasad *et al.*, 1997). The equilibrium concentrations of the α and β-ODAP corresponds to a ratio of 40:60 at a temperature of 55-60^0 C whereas at room temperature the ratio is 30:70 (Zhao *et al.*, 1999). Using NMR spectroscopy, Abegaz (1993) found that the equilibrium concentration rate of β-ODAP and α-ODAP is 3/2 at 55°C. Bell & O΄Donovan (1966) reported that β-ODAP slowly equilibrates with its isomer α-ODAP, and the inter-conversion is facilitated when heated. Zhao *et al.* (1999) reported that the equilibrium concentration ratio α- and β-ODAP decreased when the pH increased.

The α- and β- isomers differ in the position of the oxalyl moiety, Figure 3 (Yigzaw *et al.*, 2001).

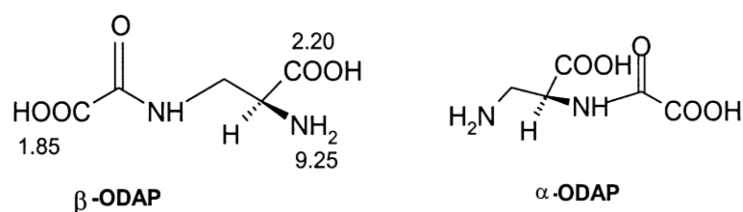

Figure 3- Structures of β-ODAP (toxic isomer), and α-ODAP (non toxic isomer), with pk_a values functional group in β-ODAP (Yigzaw *et al.*, 2001)

The levels of β-ODAP obtained in different laboratories are not always consistent. The variations can be caused by the use of different techniques such as

HPLC (high performance liquid chromatography), CZE (capillary zone electrophoresis), NIR (near infrared reflectance spectroscopy), and GC-MS (gas chromatography-mass spectrometry) (Fikre *et al.*, 2008). Some of these methods do not differentiate between α and β-ODAP. The most common method to detect ODAP utilizes the reaction of O-phthalaldehydo (OPA) with the 2,3-diaminopropionic acid (DAP) formed from hydrolysis of both ODAP α and β isomers. The derivative compound is detected at 420nm, since the free DAP does not occur naturally in the seeds of *L. sativus*. The problem is that either α or β-ODAP suffer hydrolysis making impossible to distinguish between them (Wang *et al.*, 2000).

Derivatization of 9-fluorenyl methylchloroformate (FMOC) can be used to detect and quantitatively estimate very small amounts (pmol) of ODAP, but also does not distinguish the α and β-ODAP forms. The method for pre-column derivatization with 1-fluoro-2, 4 - dinitrobenzene (FDNB) preceding the use of reverse phase HPLC, has become an important technique for the analysis of amino acids to evaluate the β-ODAP (Wang *et al.*, 2000).

On the other hand, ^{1}H-NMR spectroscopy allows the distinction of both isomers since they possess protons which are chemically and magnetically distinct and in such manner give rise to distinct resonances in the spectrum.

1.3.2.2- Grass pea detoxification

ODAP is a water-soluble amino acid that can be leached from seeds by soaking in water. Boiling or steeping the grain followed by draining out the water can reduce, considerably, the ODAP content (Mohan *et al.*, 1966; Haimanot *et al.*, 1990). Several methods have been used to decrease the ODAP levels, but none of them has been shown to be completely effective (Kumar *et al.*, 2011). In fact, soaking the grass pea seed in water can reduce the ODAP content, but not enough for safe consumption. Geda *et al.* (1995) showed a 28% ODAP reduction in whole seeds with cold water treatment for 12hr and 37% reduction with hot water (50°C) treatment for 3hr.

According to Tarade *et al.* (2007), cooking of seeds for 1hr at pH 8.0 in boiling water reduces β-ODAP content by 57% whereas pre soaking in water prior to cooking reduce β-ODAP levels up to 67%. A 1hr treatment with 1% lime water solution, sodium

chloride, sodium bicarbonate or ascorbic acid has been shown to reduce 80-90% the ODAP content in grass pea seeds (Kumar *et al.*, 2011). Fermentation, mixing the food with gravy that contains condiments with antioxidant activity or mixing with cereals rich in sulfur amino acids has also protective effects (Getahun *et al.*, 2005).

1.3.3-Neurolathyrism

As already mentioned the presence of the toxin β-ODAP, which has been claimed as the cause of a paralytic disorder known as neurolathyrism in both humans and animals, has impaired the larger cultivation and public acceptance of this plant as an edible crop (Yadav & Mehta, 1995).

Neurolathyrism is a motor neuron disease characterized by spastic paraparesis of the hind legs, without affecting sensory systems, former described by Hippocrates, (Lambein, 2012). It is different from osteolathyrism which is caused by β-aminopropionitrile (BAPN) present in seeds of *L. odoratus* (Vaz Patto *et al.*, 2011). While neurolathyrism is never lethal, osteolathyrism can cause aortic rupture (angiolathyrism).

In normal circumstances, only sporadic cases of neurolathyrism have been reported, mostly during famine and other stress conditions such as the Ethiopian famine of 1984-85, the Spanish civil war between1936-39 or at the concentration camp of Vapniarca during the World War II (Lambein, 2012). According to Kessler (1947), one of the camp prisoners that took notes about the prisoner´s diet noted that they received 400g boiled grass pea and 200g barley bread every day. Based on that, Lambein *et al.* (2001) deduced that a threshold level of ODAP intake may be between 500mg to 1g per day per adult person under the aforesaid conditions (Vaz Patto *et al.*, 2011).

Overconsumption of grass pea for an extended and uninterrupted period of time as staple food in an underbalanced diet can cause neurolathyrism (Tarade *et al.*, 2007). Although neurolathyrism can occur sporadically (Haque *et al.*, 1994), it is supposed to be epidemic in nature. Nevertheless, this crippling disease affects, preferentially, the most active young men in destitute remote rural areas and living in a hand-to-mouth economy (Lambein *et al.*, 2007).

Despite grass pea is widely consumed in large amounts around the world, the number of affected people by neurolathyrism is surprisingly low. Some Bangladeshi farm labors consume 1 Kg of grass pea seed per day, which means a daily intake of about 5g of β-ODAP, without illness effects. The variability in the susceptibility to neurolathyrism is such that no threshold of toxicity for ODAP content in the seed, grass pea intake or ODAP intake could be scientifically established (Vaz Patto *et al*., 2011). The major risks factors for the susceptibility to neurolathyrism are famine (Getahun *et al*., 2003) social stress, young age and male sex (Haimanot *et al.*, 1990; Haque *et al*., 1996), zinc deficiency (Khan *et al*., 1993; Lambein *et al*., 2007) and blood group O (Getahun *et al*., 2002b).

Environmental factors such as drought, zinc deficiency, iron or manganese oversupply and the presence of heavy metal in the soil can considerably increase the content of β-ODAP in the seeds (Lambein *et al*., 2007), as reported in Bangladesh zones where deficit of zinc occurs in the soils and in Ethiopian soils containing high levels of iron and manganese (Campbell *et al*., 1997).

The characteristic low levels of methionine and cysteine in grass pea seeds can also contribute to neurodegeneration in neurolathyrism patients by its affect on oxidative stress (Vaz Patto *et al*., 2011). Methionine is a crucial precursor for glutathione, responsible for the protection of cells (including motor neurons) against oxidative stress.

1.4- *Lathyrus sativus* L. tissue culture

In recent years, biotechnological tools have been used to improve the quality of cultured plants. In the case of *L. sativus*, the work carried out to improve this species remains scarce. Few groups have developed *in vitro* approaches with *Lathyrus* species (Ochatt & Jain, 2007). In (1965) Pecket & Selim reported the *in vitro* culture of *L. sativus* embryos from which calluses were obtained. Shoot multiplication from shoot apices and stem calli were also reported (Mukhopadhyay & Bhojwani, 1978; Sinha *et al*., 1983) but little practical progress was made in these early experiments. However, Roy *et al*. (1991) reported the *in vitro* production, by organogenesis, of plants from cultured leaf discs, root (1992) and internodes (1993). Malik *et al*. (1993) reported *in vitro* shoot bud differentiation or plant regeneration via callus derived from direct shoot

regeneration from epicotyls explants and seed cultures. Mehta *et al*. (1994) obtained somaclones of *L. sativus* from leaf, internode and root-derived callus cultures, which in several phenotypic characters, including ODAP levels, were largely stable over three generations.

Rooted plants were difficult to produce and it has been seen that shoots grew on the callus surface probably developing from pre-existing meristems (Ochatt *et al.,* 2002). Fratini & Ruiz (2003) described an efficient rooting procedure for *in vitro* regenerated shoots of several cool season grain legumes including *L. sativus*.

Ochatt *et al*. (2002) obtained some regenerants with a normal phenotype and DNA whereas somatic hybrid calli were produced following leaf fusion of protoplasts of grass pea and pea (Yan *et al*., 2006). The availability of these regeneration techniques in the production of fertile plants may help the breeding of *L.sativus*, and the regeneration of somatic hybrids might also yield genotypes with the disease resistance from grass pea coupled with the grain quality from other leguminous crops (Yan *et al*., 2006).

Only after 2000 year, fertile plants efficiently and prolifically regenerated from meristematic tissues (Zambre *et al*., 2002), from hypocotyls segments (Ochatt *et al*., 2002) and from epicotyls segments (Barik *et al*., 2005). At the same time that progresses on the development and exploitation of biotechnology tools for breeding grass pea were achieved, other studies indicated that it was possible to reduce the generation cycles (Ochatt *et al*., 2004). The results obtained from three different *L. sativus* genotypes over 10 successive generation cycles showed a shortening of each cycle from the standard 150-180 days in the field to 100-120 days *in vitro*, as resumed by Vaz Patto *et al*. (2011).

Zambre *et al*. (2002) developed a new *in vitro* protocol for prolific shoot regeneration from two elite genotypes of *L.sativus*: vegetative apical and axillary bud explants from greenhouses-grown plants were used to induce regeneration-competent nodular callus. According to Yan *et al*. (2006), the regeneration method based on green nodular callus is considered the most efficient amongst the available protocols for obtaining fertile plants of *L.sativus*. This will allow exploit grass pea lines with the

objective of improving yield potential, adaptability and nutritional quality through reduction of β-ODAP, for human consumption and animal feed.

1.5- *Lathyrus sativus* L. genetic diversity

Genetic studies on *Lathyrus* spp. are not very common. Detailed chromosome analysis showed that grass pea has a chromosome number of 2n=14, with two metacentric and five submetacentric chromosomes (Kumar & Dubey, 1996b).

Significant repositories of germplasm of *L.sativus* are held in India at the National Gene Bank (New Delhi) at ICARDA in Syria (Arora *et al.*, 1996; Abd El-Moneim *et al.*, 2001), providing access to a wide range of agro-morphological traits including lines with low ODAP content (Hillocks & Maruthi, 2012). The widest collection of *Lathyrus* species is held under the European Cooperative Program on Crop Genetic Resources, hosted by Biodiversity International in Rome, which has over 4.000 accessions (Hillocks & Maruthi, 2012). ICARDA also holds a large collection of *Lathyrus*, containing 3.300 accessions, Russia holds 1.240 accessions, Australia has over 1.000 accessions and Algeria has 463. Among European countries Germany, Hungary and Spain hold collections with 300-500 accessions. The largest of *Lathyrus* in Africa is held by Ethiopia with 96 accessions. With respect to *L. sativus,* France has the largest collection, with 4.387 accessions. The Indian national Genebank contains 2.720 grass pea accessions and Bangladesh has 2.078 (Campbell, 1997; Kumar *et al.*, 2011).

ICARDA holds *Lathyrus* germplas from more than 45 countries under the auspices of Food and Agriculture Organization (FAO). The majority of accessions of all species of *Lathyrus* held in ICARDA, except *L. sativus*, are from Morocco, Algeria, Tunisia, Turkey, Syria and Jordan. The *L. sativus* accession is from Ethiopia, Bangladesh and India (Robertson & El-Moneim, 1996). The main utilization of ICARDA germplasm collection is crop improvement of *Lathyrus* with the objective to produce varieties with improved quality by reducing β-ODAP content and improving the palatability, intake and nutritive value of herbage and seed.

Broadly, grass pea germplasm can be divided into two groups (Benkova & Zakova, 2001); one from Asia with small seeds and average high ODAP content and

other from the Mediterranean basin with bigger seeds and lower average ODAP content. European germplasm can be subdivided into two further groups based on morphological characters. Accessions from Spain and Italy were found to be short with large seeds, while those from west-central Europe (France, Germany and Poland) were taller with smaller seeds (Grela *et al.*, 2010).

1.5.1- Diversity analysis

There is a rising concern about the genetic erosion of *Lathyrus,* mainly in Mediterranean area, and some *ex situ* conservation actions were suggested by the International Board for Plant Genetic Resources (Shehadeh, 2011). Nevertheless, to achieve this aim, it is imperative the detailed characterization of the existing diversity (Vaz Patto *et al.*, 2011), since the evaluation of the genetic diversity is a preliminary steep that ensures the conservation and improvement of genetic resources. Consequently it is important to know which are the most *L.sativus* related species, since these are the most promising source of traits.

A study of genetic diversity and its relation to geographical diversity may give information about the center of diversity and origin of domestication of a cultivated crop. Issues, like whether or not genetic variation is being lost with progressive domestication or how the variation is distributed among populations, can be also addressed by studies of genetic diversity (Chowdhury & Slinkard, 2000).

The identification of the species closely related with *Lathyrus* has been carried out mainly supported on morphological and phylogenetic studies. The profound interspecific hybridization barriers in the genus *Lathyrus* are confirmed with the results of some electrophoretic patters of the total seed protein globulin A (El-Shanshoury, 1997; Przybylska *et al.*, 1999), showing a considerable phylogenetic distance between *L. sativus* and the other examined species. On the other hand, molecular studies, morphological analyses, and interspecific hybridization have detected a close phylogenetic proximity between *L. sativus* and *L. cicera* (Jackson & Yunus, 1984; Croft *et al.*, 1999; Ben Brahim *et al.*, 2002; Emre, 2009; Shiferaw *et al.*, 2012). *L. sativus*, being an annual species and with high levels of autogamy (Chowdhury &Slinkard,1997)

presented low levels of genetic diversity and a low level of gene flow when compared with other *Lathyrus* species (Vaz Patto *et al.,* 2011).

The exploitation of germplasm resources for the improvement of grass pea must concentrate on the primary gene pool, including wild and landraces materials. According to Vaz Patto *et al.* (2011) there is a high improvement potential in this material since high variability has been found at the primary gene pool within *L.sativus* accession. *L.sativus* is placed in section *Lathyrus* among other 33 species. Based on crossability and cytological evidence between grass pea and 15 wild species in the section *Lathyrus*, Yunus & Jackson (1991) placed *L.cicera* and *L.amphicarpos* in the secondary gene-pool and the other species in the tertiary gene-pool of grass pea.

By studying a collection of 49 different accessions from all over the world, Jackson & Yunus (1984) showed that grass pea is differentiated into distinct forms, such as flower color, seed size and size of leaves.

In 1999 Hanbury, as resumed by Vaz Patto *et al.* (2011) evaluated under Mediterranean-type environments, a worldwide collection of *L. sativus* accessions, and divided them into two clusters based on the phenology, seed yield and ODAP content. This division matched their geographical origins: Indian subcontinent and Mediterranean/European. The Mediterranean/ European origin lines were high yielding, with larger seeds and later flowering.

Tavoletti *et al.* (2005) on a more restricted germplasm study using a collection of 16 potential grass pea landraces from the Marche region (Italy) found a high level of phenotypic variability between commercial and household populations.

Diversity among and within populations has been detected for several of the analyzed traits showing the high breeding potential of these materials and that these traits can be improved further through a strong selection program (Vaz Patto *et al.*, 2011).

1.5.2- *Lathyrus sativus* L. breeding – what has been achieved

Despite the many advantages that the grass pea presents, relatively few studies has been developed for the improvement of *L. sativus,* a culture underutilized. Some

efforts were started in India, Canada, Bangladesh, Ethiopia and Nepal, to promote the breeding, in the late seventies (Kumar *et al*., 2011). The Center for Agricultural Research for Dry Areas (ICARDA) only in 1989 began to develop programs for the breeding of this crop. The major grass pea improvement programs have been conducted in India, Bangladesh, Australia, Ethiopia and at ICARDA in Syria (Hillocks & Maruthi, 2012).

Several laboratories around the world have already succeeded in releasing lines with levels of β-ODAP, which represents about 1% of the original wild type (0.5% - 1.5% down to 0.01% or less) (Vaz Patto *et al*., 2006). For instance the breeding program established in Canada has released a germplasm LS8246 with 0.03% of seed ODAP content (Yan *et al*., 2006). Breeding programs were developed in order to obtain lines that combine low ODAP and simultaneously high yield (Kumar *et al*., 2011).

In Portugal a number of different studies have been recently initiated in *L. sativus*. Vaz Patto *et al*. (2007) identified a range of resistance reactions to powdery mildew in a collection of Iberian *L. sativus* germplasm, also including Portuguese accessions. Also the identification and characterization of partial resistance to rust in the same germplasm collection of *L. sativus* was addressed (Vaz Patto & Rubiales, 2009). From these accessions, resistant plants were selfed and crossed with susceptible accessions. The obtained progeny is being used to study the inheritance of the detected resistances. Almeida *et al*. (2011, 2012, 2013) developed several different types of molecular markers, specific to *L. sativus* and *L. cicera*, which will allow future molecular studies such as linkage analysis and diversity studies. Several authors (Sardinha *et al*., 2007; Almeida *et al*., 2009), have studied the genetic diversity of this Iberian *L. sativus* germplasm collection using AFLPs, but no clear clustering was detected based on this molecular data. Silvestre *et al*. (2013 submitted) addressed the resistance mechanism to water stress using new and self developed tools. In a more classical agronomical study, Carita (2012) analysed the effect of sowing date and seeding density on yield of four genotypes of *L. sativus*.

1.6 - Objectives

This work is part of a broader objective which the main purpose is to develop genotypes of *L. sativus* more productive and with lower β-ODAP content. This long term objective can only be achieved after a better understanding of the genetic diversity of *L. sativus*, the establishment of effective protocols of plant propagation to obtain large amounts of plants genetically uniform and the development of effective methods to detect the among of β-ODAP on seeds and how this compound is affected by cooking. In this work, we have only worked with plant material originated from Serra do Sicó, a small area in the center of Portugal. However, there are other regions in Portugal where *L. sativus* is also cultivated and which must be used in future studies. Considering this main goal, this work has three main purposes, namely:

1- To establish a micro-propagation protocol for several *L. sativus* landrace populations.
2- To study the genetic diversity and population structure among eight Portuguese *L. sativus* landrace populations, in order to understand the present diversity status and acquire information that will contribute to ensures the conservation and the development of genetic resources for future breeding programs.
3- To identify and quantify the β-ODAP content from the eight different *L. sativus* populations. Preliminary studies about the kinetics of β-ODAP conversion to α-ODAP were also carried out.

Due to the diversity of the methods used, this work was performed in three different laboratories:

1- Plant Biotechnology Laboratory – Departamento de Ciências da Vida da Universidade de Coimbra.
2- Plant Cell Biotechnology Laboratory - ITQB (Instituto de Tecnologia Química e Biológica)/ Universidade Nova de Lisboa.
3- NMR Laboratory – Centro de Neurociências/ Departamento de Ciências da Vida da Universidade de Coimbra.

2. MATERIALS AND METHODS

2.1 – Micropropagation protocol for *Lathyrus sativus* L. Portuguese landraces

2.1.1 – Plant material

The seeds were collected locally during the 2012 year, in June, from eight farmers: four from Alvaiázere county and other four from Penela county, as indicated on Table III.

Table III- Origin of the *L. sativus* populations used

Population	Main origin	Local origin	GPS coordinates
LS1	Alvaiázere	Pussos	Lat:39º47´55.31"N Lon:8º21´28.24"W
LS2	Penela	Rabaçal	Lat:40º1´42.68"N Lon:8º26´55.72"W
LS3	Alvaiázere	Venda do Preto	Lat:39º47´19.19"N Lon:8º26´55.72"W
LS4	Alvaiázere	Chancas	Lat:39º48´23.04"N Lon:8º20´39.53"W
LS5	Penela	S. Sebastião	Lat:40º0´54.56"N Lon:8º24´55.67"W
LS6	Alvaiázere	Venda do Preto	Lat:39º47´35.69"N Lon:8º25´05.27"W
LS7	Penela	S. Sebastião	Lat:40º1´06.14"N Lon:8º24´41.62"W
LS8	Penela	Chão de Ourique	Lat:39º59´02.00"N Lon:8º24´04.32"W

2.1.2 – Seed sterilization

Seeds were surface sterilized by treating them with ethanol 70% (v/v) for 1 min, followed by a treatment with mancozeb (Sapec Agro, Setúbal, Portugal) solution (2g/L) under agitation for 30 min. After this treatment seeds were rinsed three times with distilled water and then immersed for 15 min in a 7 % (w/v calcium hypochlorite solution plus two drops of "Tween-20" (polyoxyethylenesorbitan monolaurate; Sigma Chemical Co, St Louis, USA). Finally, seeds were rinsed again three times with autoclaved distilled water in a laminar flow hood.

2.1.3 – Seed germination

Seeds were placed in 120 cm^3 autoclaved screw-capped jars containing watered cotton covered with. A total of 100 seeds randomly chosen (20 seeds/ population) were placed in jars each one containing five seeds. Jars were placed in the greenhouse at temperature of 25^oC under 16 hour daily illumination and seeds periodically checked.

2.1.4 – Shoot proliferation

Shoot tips (5 mm length) from developing seedlings (9 days) obtained in the conditions indicated in the previous section were isolated and cultured on a half-strength MS (Murashige & Skoog, 1962) medium with 3% (w/v) sucrose and 0.6% (w/v) (Panreac, Spain). One µM benzylaminopurine (BAP) was added and the pH of the medium adjusted to 5.7 – 5.8 with HCl (0.1-1N) or KOH (0.1-1N). The medium was autoclaved at 120 ^oC (1.1 atm) for 20 min.

A total of 14 shoot tips per population (Table III) were cultured in test tubes (two explants per tube) as shown in figure 4. The cultures were maintained in the same conditions as described earlier for seed germination. After two weeks, the number of nodes per explant, number of shoots and shoot length were recorded.

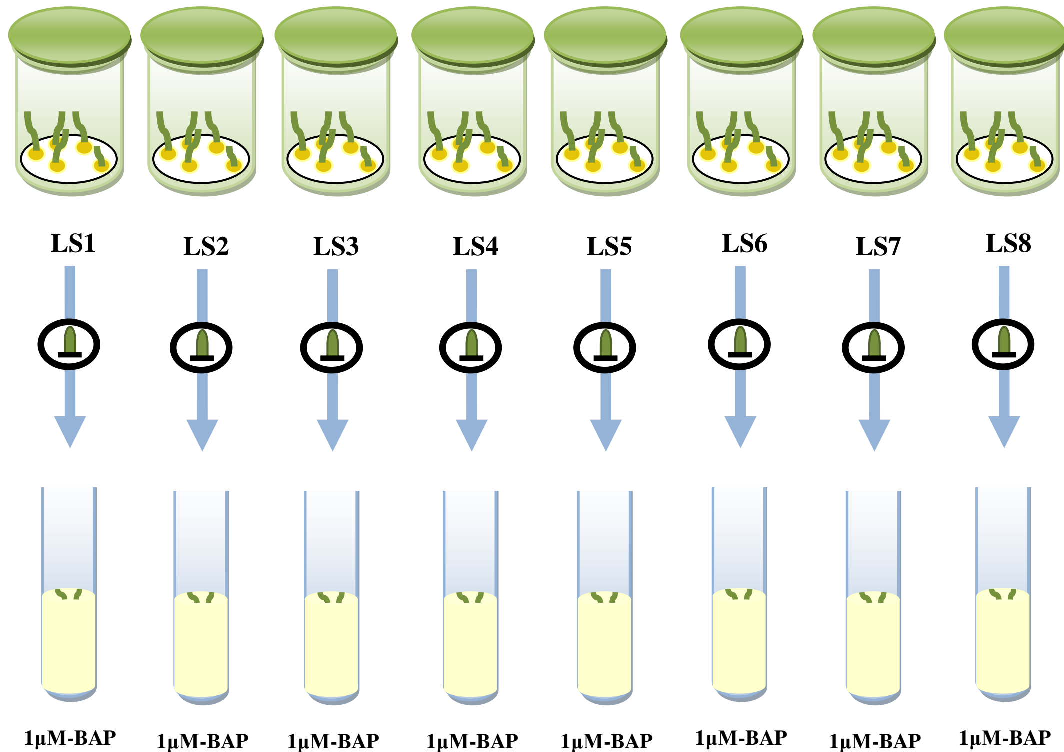

Figure 4 – Experimental procedure for shoot tip culture of different genotypes of *L. sativus*.

In a second experiment (Fig. 5), shoot tips from the genotype LS2 were obtained in the same conditions as described before and tested on culture media containing different concentrations of BAP (0, 1, 2, 4, 8 and 10 µM). Media preparation and culture conditions were the same as before. A complete randomized design with three replicates per treatment and 20 explants per replicate was used for each BAP concentration. After five weeks, the number of nodes per explant, number of shoots and shoots length were recorded.

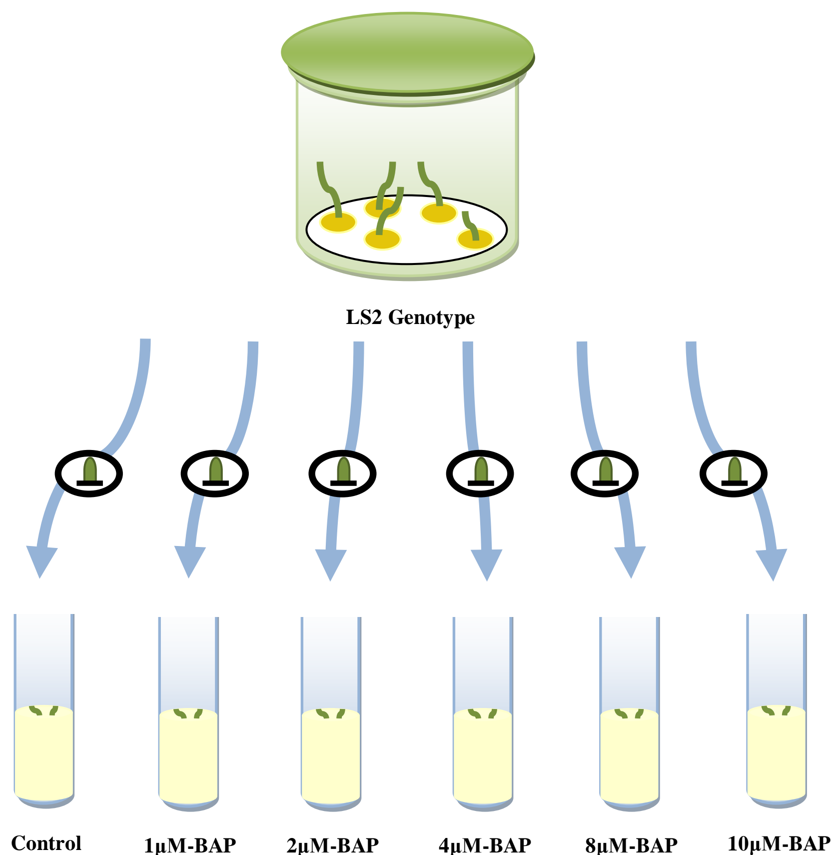

Figure 5 - Shoot tip culture of the genotype LS2

2.1.4.1- Plant material

Shoots (2 – 3 cm length) obtained from shoot tip culture on media containing 2 or 10 µM BAP were used in rooting assays. Shoots 5 mm were removed from the proliferation media and transferred to the same basal medium containing 2.46 µM IBA (indole-3- butyric acid), 2 % (w/v) sucrose and gelled with 0.6% (w/v) agar. A medium without IBA was used as control. A total of 36 explants from each proliferating medium were tested in rooting media. Thus, the total number of shoots tested was 144: 2 proliferation media (72) x 2 rooting treatments (Fig. 6). Rooting treatments were carried out at 25ºC in the dark. After 2 weeks in the rooting medium, explants were transferred to the same media without IBA for root expression. After 1 month in the expression medium, the number of shoots producing roots was recorded.

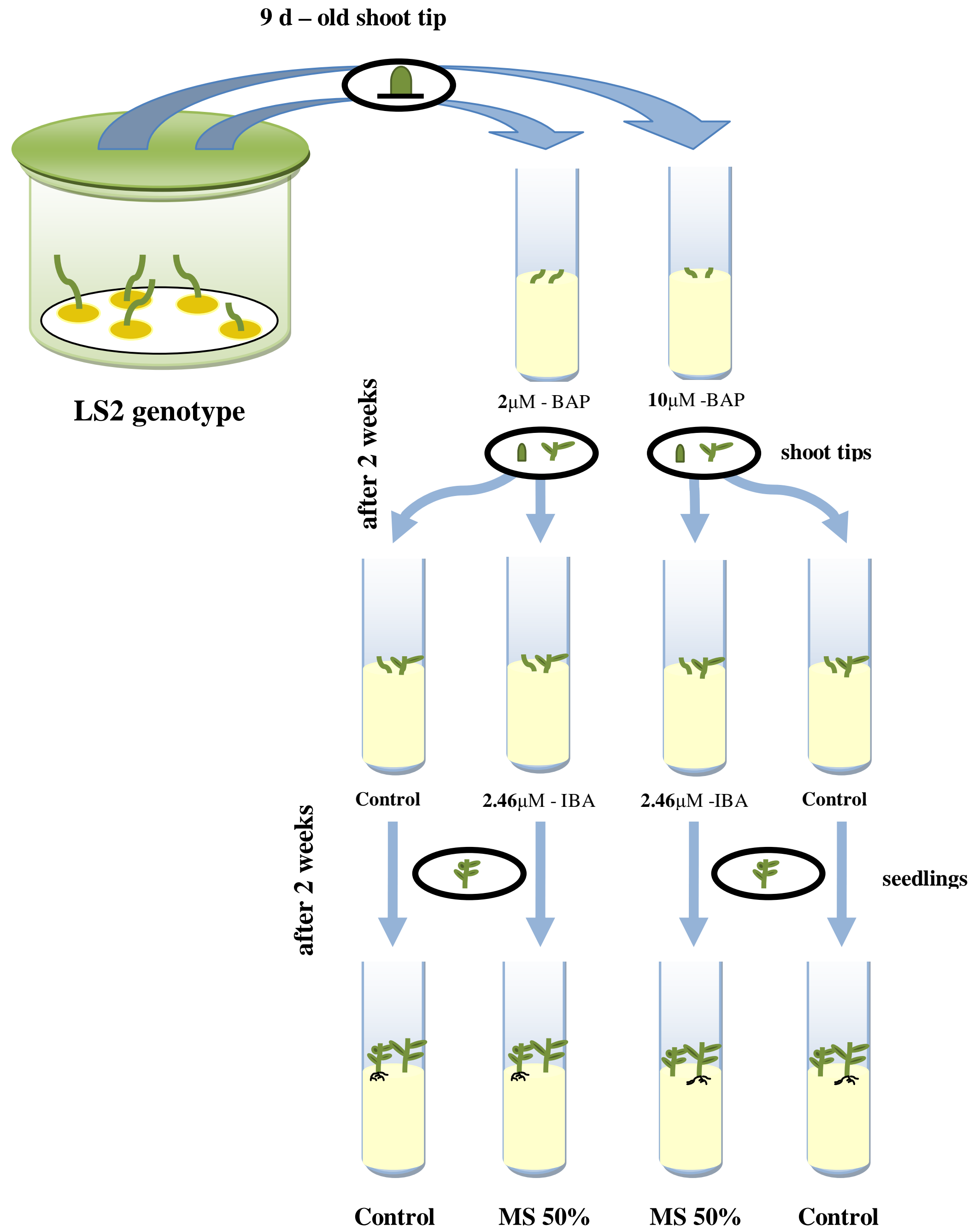

Figure 6 – Protocol used for *L. sativus* rooting.

2.1.5- Statistic analysis

The data concern to number of nodes were analyzed with Statistic 7.0 using one-way ANOVA and the post hoc tests were performed using a Tukey test for a significant level $p<0,05$.

2.2- Diversity study of eight *Lathyrus sativus* L. Portuguese landraces.

2.2.1-Plant material

The analysis was carried out on eight populations of *L. sativus* described on Table III.

Seeds (40) from each of the populations were sowing in pots (4 seeds per pot). The pots were placed in a growth chamber at a 20ºC-22ºC temperature at night and 25ºC-27ºC during day under a 12hr photoperiod and daily watered. Four weeks later eight to ten young leaves from each plant were harvested and put into Eppendorf tubes (two tubes for each individual), properly identified, immediately submerged in liquid nitrogen to prevent DNA degradation and finally .They were stored at -80° C.

The total number of plants collected ranged from 27 to 30 of each population, according to Table IV.

Table IV- Number of total collected samples / population

Population	LS1	LS2	LS3	LS4	LS5	LS6	LS7	LS8
Nº of plants	27	27	30	27	28	30	30	27

Eighty individuals randomly chosen from the eight populations (LS1, LS2, LS3, LS4, LS5, LS6, LS7 and LS8), 10 individuals per population, were selected for the subsequent DNA isolation and to assess the intra and inter specific variation levels of diversity.

2.2.2 - DNA isolation

Total genomic DNA from fresh young leaves was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol developed by Torres *et al.* (1993).

Two or three leaves were placed into a 2 mL tube, together with three tungsten balls, a pinch of sodium bisulfite, a pinch of polyvinylpyrrolidone (PVP) and two pinches of sand. The samples were grounded in a mill Tissue lyser MM300, Retsch (Conquer Scientific, San Diego, USA) using three cycles of 30's at 30r/s each. After each cycle,

samples were dipped in liquid nitrogen to prevent thawing and subsequent DNA degradation.

One mL extraction of buffer 2% CTAB + 0.4% mercaptoethanol was added to each sample, stirred on a vortex mixer, IKA vortex Genius 3 (IKA, Wilmington, USA) and placed in a water bath at 65° C for 30 minutes. This step was performed on the fume hood. Mercaptoethanol is a strong reducing agent which can remove tannins and other polyphenols often present in the crude plant extract.

Samples were removed and added to 900 µL of 24:1 chloroform: octanol. This step and the two next steps were also performed on the fume hood. Gently stirred for 10 minutes and centrifuged for eight minutes at 10.000 rpm using a Eppendorf centrifuge 5424 (Hamburg, Germany).

The supernatant was transferred to a new 2 mL tube. Repeated addition of 900 µL of 24:1 chloroform: octanol, stirring 10 minutes and eight minutes centrifugation at 10.000 rpm.

The supernatant was transferred to a 1.5 mL tube and added 1.000 mL of isopropanol, to link DNA and precipitate it.

The samples were placed at -20° C for one hour. After this time, were centrifuged for eight minutes at 12.000 rpm.

The supernatant was discarded, 1mL of wash buffer (75% ethanol and 0.2 M sodium acetate) was added and samples were left for one hour at room temperature.

The supernatant was then discarded and the samples were placed into freeze drying Speed Vac Plus SC110A, Savant (GMI Icn, Minnesota, USA) for complete drying.

One hundred mL of a dilution buffer TE pH 8.0 were added and the samples left overnight at 4° C. The next step was the addition of 0.5 mL / sample RNAse (stock concentration 10 ng/µL) and held in an oven at 37° C for 45 min.

DNA quantification and purity were performed in a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), using 1.5 mL / sample.

DNA size and integrity were determined by gel electrophoresis in1% (w/v) agarose dissolved in 0.5X TBE (pH 8.3) according to the following protocol:

One gram of agarose was weighed and dissolved into 100 mL of 0.5X TBE (pH 8.3). The mixture was heated in microwave for 1.15 minutes at 750 watts. Cooled to about 40 $^{\circ}$C and added with 1µL of "Syber Save" (ethidium bromide used to staining the DNA for visualization under UV light). The solution was poured into the cradle, the comb was placed and allowed to polymerize adequately protected from light.

In a 96-well microtiter plate 6 µL of “1x Green Go Taq Reaction buffer" + 1µL of each sample were load. Two reference markers, λ50 ng and λ200 ng, were used which were also mixed with the "1x Green Go Taq Reaction buffer" which enabled to compare the estimated size of DNA.

Using a multichannel pipette, the agarose gel was loaded, by order, with 7µL sample + " 1X Green Go Taq Reaction buffer " and placed in a submarine electrophoresis system Mupid-exu (Advance, Japan) filled with 0.5 x TBE and made to run for 20 minutes at 100 V.

The stained DNA was visualized in a GEL-DOC 1.000 system (Bio-Rad, Hercules, USA) with ultraviolet light.

All samples were diluted with TE in order to obtain a "stock solution" at a concentration of 100ng/µL. Subsequently, further dilutions with TE were made to obtain a “working solution” at a concentration of 10ng/µL.

2.2.3- Polymerase chain reaction (PCR) – EST-SSR amplification

Five pairs of expressed sequence tag microsatellite (EST-SSR) markers were used. These EST-SSRs markers were obtained previously by Almeida *et al*. (2011)by searching for microsatellite motifs with the Phobos software (Mayer, 2010) in *L. cicera* cDNA libraries (Almeida *et al*., 2011) using the following selection criteria: 1) cross-species amplification in *L. sativus*; 2) polymorphism in *L. sativus*; 3) easy band scoring on polyacrylamide gel (Almeida *et al*., unpublished). Microsatellite characteristics are summarized in Table V.

Table V- Characteristics of the five EST-SSR markers used. Range size and nº of alleles values concerning to the eight populations studied.

Primer	Microsatellite motif	Repeat motif	Size range (bp)	Allele nº.
S037-011	$(GAA)_7$	3	212-221	4
S037-022	$(GGT)_5$	3	113-116	2
S037-035	$(GTCGCC)_3$	6	216-228	2
S132-040	$(CAC)4CGA(CGC)_3$	3	273-288	3
S132-100	$(ATGTTG)ACG(ATGTTG)_3$	3	254-260	2

PCR amplification was performed according to the following procedures and conditions (optimized by Almeida *et al.*, 2013):

Each reaction was conducted in a total volume of 10 µL containing 10 ng of template DNA, 0.04 µM of M13(-21) tagged forward primer - an M13 tail was added to the 5` -end of the forward primers, allowing them to be labeled IRD fluorescence (Schuelke 2000), to allow resolution using a LI-COR 4300 DNA Analyzer (Lincoln, NE, USA) - 0.16 µM of reverse primer (Eurofins MWG Synthesis GmbH), 0.16 µM of IRD 700 or IRD 800 M13(-21), 1.5 mM of $MgCl_2$ (Promega, Madison,USA);, 0.2 µM of each dNTP (Invitrogen) and 0.2 units of Taq DNA polymerase (Promega, Madison,USA).

The amplification reaction was performed in a Biometra Uno II thermal cycler, under the following amplification profile: initial denaturation of 5 min at 94 °C, followed by 30 cycles with denaturation of 30 s at 94 °C, annealing of 45 s at 56 °C 45 s at 72 °C. Followed eight cycles with annealing of 3 s at 94 °C, 45 s at 53 °C and extension of 45 s at 72 ºC. The reaction was terminated with final extension at 72 °C for 10 min.

The amplified fragments, 5 µL, were resolved using 0.8% (w/v) agorose gels in 0.5 X TBE buffer, performed as described above. Three µL markers (ladder) DNA 100bp were used as reference.

Electrophoresis of the amplified fragments was carried out using a submarine electrophoresis system Mupid-exu (Advance, Japan) at100V for 25 min;

It was performed an electrophoresis using a submarine electrophoresis system Mupid-exu (Advance, Japan) at 100V for 25 min.

Gels were stained with ethidium bromide and visualized on GEL-DOC 1000 System (Bio-Rad, Hercules, USA) under UV light.

2.2.4- Genotyping

A vertical electrophoresis was performed in 4300 DNA Analyzer LI-COR Version 2.0 (Lincoln, NE, USA), using a 6.5% polyacrylamide gel.

Sample preparation was performed according to the following procedure.

In a PCR 96-well microtiter plate, were mixed 25µL FLB -Formamide Loading Buffer- containing 95% of formamide, 20 mM EDTA and 0.8 mg / mL bromophenol blue) with 1µL PCR product, in all wells corresponding to samples.

All the empty wells of the PCR plate were loaded with 25µL TE.

The plate was shaken for 5 min at 600 rpm, using a MS1 Minishaker (IKA, Wilmington, USA) and centrifuged during 6-8 s at 2500 rpm on a Sartorius 4K15 (Sigma, Osterode, Germany).

Samples, as well as phytochrome used were denatured at 95° C for 5 min in thermal cycler UNOII (Biometra, Goetting, Germany).

After this period, phytochrome and the samples were immediately placed on ice and protected from light.

Using a Hamilton 8-channel 0.2 mm syringe (LI-COR, P/N 870-05848), 0.7 µL were loaded in a 6.5% KB Plus (20 µL of 6.5% Gel Matrix, 150 µL APS 10% and 15 µL TEMED) denaturing polyacrylamide gel in a LI-COR 4300 DNA analyzer. Also 0.3 µL of DNA size standard with a range from 50 bp to 350 bp or 50 to 700 bp were loaded in five wells evenly distributed in the gel. Electrophoresis took place under the

following conditions: voltage 1500 V, power 40W, current 40 mA, temperature 45ºC and speed 2. Allele scoring was done using the SAGA Generation 2 software, from Li-Cor, as presence or absence of each marker allele and this information was after transferred to an Excel file.

2.2.5- Data Analysis

For the data analysis only individuals with less than 60 % of missing value were considered, therefore eight individuals were removed from the analysis (four individuals of population LS3, two individuals from LS4, one individual from LS5 and one individual from LS7).The excluded individuals presented more than 60% of missing value because they did not amplified during the screening process, thereby the number of scored alleles was very low and the results could biased the data analysis. The effective number of individual per population used for data analysis is described in table VI.

Table VI- Number of total individual / population used for data analysis

Population	LS1	LS2	LS3	LS4	LS5	LS6	LS7	LS8
Nºof individuals	10	9	6	8	9	10	9	10

The eight populations of *L. sativus* under study were genotyped with five polymorphic *L. cicera*-derived EST-SSRs markers (Table V).

Polymorphism Information Content, (*PIC*; Botstein *et al.*, 1980) of each microsatellite marker was calculated by PowerMarker V3.23 (Liu, 2002) software. The PIC value of each marker was used to assess their informativeness, which is determined by the number of alleles and their frequency distribution within a population.

GENEPOP 4.0 (Raymond & Rousset 1995) was used to calculate (1) the observed heterozygosity (H_O) as the effective number of the heterozygotes observed, and (2) the expected heterozygosity (H_E) as the (expected) probability that an individual will be heterozygous at a given locus. It was also used to calculate (3) the inbreeding coefficient (F_{IS}) as the proportion of the variance in the subpopulation contained in an individual.

The probability test was based on Markov chain method (Guo & Thompson 1992; Raymond & Rousset, 1995). The sequential Bonferroni adjustments (Rice, 1989) were applied to correct for the effect of multiple tests using SAS Release 9.2 (SAS Institute, 2004). The average number of alleles per locus (N_{av}) and the allelic richness, N_{ar}, as the measure of the number of alleles per locus independent of sample size was calculated by FSTAT v. 2.9.3.2 program package (Goudet, 2002) while the number of private alleles (N_{pr}) per population was assessed by GDA ver. 1.0 (Lewis & Zaykin, 2001).

FSTAT was used also for testing the significance of differences in average values of N_{ar}, H_O, H_E and F_{IS} (A) between populations from Alvaiázere and Penela.

Genetic differentiation between all pairs of populations was measured with pairwise F_{ST} estimates. Pairwise F_{ST} and their respective *P*-values for significant differences from zero were calculated in FSTAT. FST is a primary metrics utilized for empirically estimating and testing the magnitude of genetic divergence among populations.

A factorial correspondence analysis (FCA) was carried out using Genetix 4.05 (Belkhir *et al*., 2004) in order to represent genetic relationships among individual plants.

The proportion-of-shared-alleles distance (DPSA; Bowcock *et al*., 1994) between pairs of accessions was calculated using MICROSAT (Minch *et al*., 1997). Cluster analysis based on distance matrix was performed using the Neighbor-Joining algorithm as implemented in NEIGHBOR program of the PHYLIP ver. 3.6 software package (Felsenstein, 2004). The reliability of the tree topology was assessed via bootstrapping (Felsenstein, 1985) over 1000 replicates generated by MICROSAT and subsequently used in NEIGHBOR and CONSENSE programs in PHYLIP.

The analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was used to partition the total microsatellite diversity (1) among and within *L. sativus* populations, (2) between the origin of the populations (Alvaiázere *vs*. Penela), among populations within different origins and within populations. The variance components were tested statistically by non-parametric randomisation tests using 10,000 permutations in ARLEQUIN ver. 3.0 (Excoffier *et al*., 2005).

2.3 - β-ODAP identification and quantification NMR Spectroscopy.

2.3.1 – Plant material

The same eight genotypes of *L. sativus* Portuguese landraces seeds (Table III), collected from seven household farmers and one market-oriented farmer in Alvaiázere and Penela, were used.

2.3.2 - β-ODAP standard synthetic formula

The β-ODAP standard synthetic formula was used as a pattern to identify the β-ODAP present in our extracts. It was obtained from Dr. Rao S L N Company (Lathyrus Technology, Hyderabad, India).

2.3.3 - β-ODAP extraction

Beta-ODAP was extracted using a modified protocol described by Wang *et al.* (2000). Dry seeds were powdered in a mortar with liquid nitrogen. The seed powders were stored at 4°C. Twenty mg of each sample accurately weighed were added to 2 mL of ethanol-water (3:7, v/v) in a small beaker, shaken briefly and agitated with a magnetic stirrer for two hours. To maintain low temperature, the beakers were placed into ice, during the agitation process. Samples were transferred into a 2 mL tube and centrifuged for 15 min at 15.000 rpm. The supernatant was collected and the placed at 4°C, overnight with the tube cover open. The samples were placed into a freeze drying, model Freezone Centri Vap Concentrator (LABCONCO, Kansas City, USA) until complete drying.

2.3.4 – Sample preparation

2.3.4.1 - β-ODAP identification and quantification

The lyophilized powder resulting from ethanol: water extraction procedure was dissolved in 0.2 mL D_2O (99.9% in D) for performing proton (^1H) Nuclear Magnetic Resonance (NMR) spectroscopy. A sample of standard β-ODAP synthetic formula was also prepared for identification purposes. Spectra were acquired on a 600 MHz Varian

(Palo Alto, CA) NMR spectrometer, using a 3 mm indirect detection probe. Typical acquisition parameters included a 45° radiofrequency (rf) pulse, a 3 seconds acquisition time and an interpulse delay of 10 seconds to allow full relaxation of all nuclei in the sample, for purposes of quantification. Spectral deconvolution was made by the NMR processing software NUTSpro (Acorn, Fremont, CA), using the line-fitting subroutine. Before Fourier transformation free-induction-decays (FIDs) were multiplied by a 0.5 Hz Lorentzian to improve signal to noise ratios.

2.3.4.2 – Kinetics of β-ODAP conversion into α-ODAP by thermal isomerisation.

For kinetics of β-ODAP isomerisation were used both the standard synthetic formula and the population LS4 that showed the highest level of β-ODAP.

Five tubes with 20 mg each of seed powder were prepared and the β-ODAP extracted according to the protocol described above. To each dry extract sample were added 200 μL of D_2O.

At the same time five samples consisting of 2 mg of β-ODAP dissolved in D_2O were prepared.

The 10 samples were used for a kinetic thermal analysis of β-ODAP isomerisation into α-ODAP. Two, one from each set, were not subject to boiling water (100°C) and the other 8, were subject to different time intervals in boiling water: 10, 20, 30 and 60 minutes. At the end of each time period samples were withdraw and immediately placed in ice.

^{1}H-NMR spectra were collected for each of the 10 samples, using the acquisition parameters described above, allowing a kinetic evaluation of the conversion of β-ODAP into α-ODAP.

3. RESULTS

3.1– Micropropagation of *Lathyrus sativus* L. Portuguese landraces.

3.1.1 - Evaluation of the regeneration response of the eight populations of *Lathyrus sativus* L.

To evaluate the potential of the different genotypes the following parameters were recorded: 1) number of nodes, 2) number of shoots, and 3) shoot length.

When the first parameter was analysed it could be observed that LS2 originated the highest (4.14) average number of nodes (4.14) followed by LS4, with an average of 4.0 nodes per explants (Fig. 7 –A). In all the other genotypes the response was lower but statistical significantly differences among genotypes were not found (Fig. 7-A). Similar results were obtained when the average number of shoots was the parameter analysed (Fig. 7 –B). However, average shoot length was higher on the LS1 genotype (Fig. 7 – C).

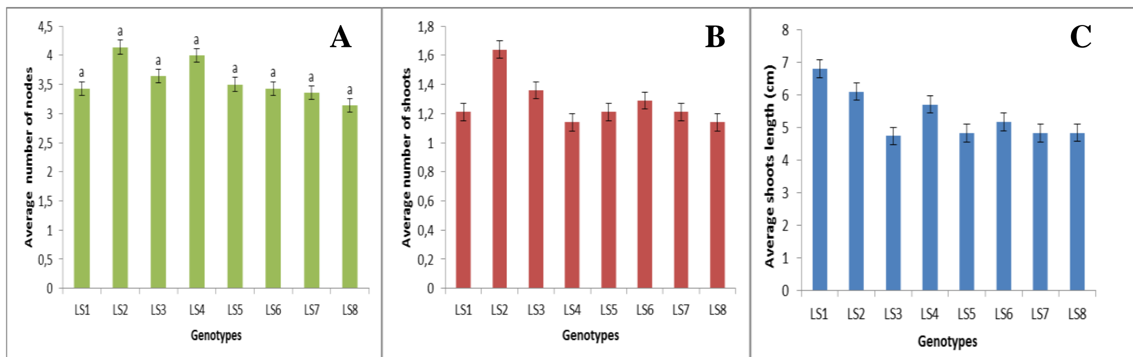

Figure 7 – Number of nodes (A), average number of shoots (B) and average shoots length in shoot tip cultures of 8 genotypes (LS1-LS8) of *L. sativus* on a medium containing 1µM BAP. The results were taken after five weeks of culture. Values are the mean ± SE of three replicates and those followed by the same letter are not significantly different at the P<0, 05 level using the Tukey test.

Since the LS2 genotype showed the highest number of shoots and nodes (Fig. 8) it was chosen to perform the next experiments.

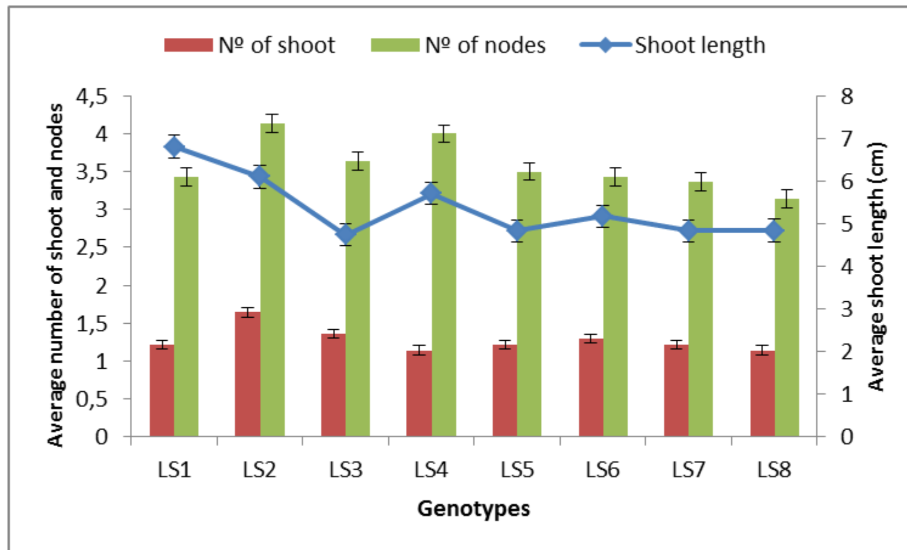

Figure 8- Comparisson between the response of the different genotypes to shoot tip culture.

3.1.2 - Effect of BAP on LS2 genotype shoot tip culture.

In order to find the best concentration of BAP to promote *L. sativus* micropropagation, five different concentrations of BAP and a control (without BAP) were tested using the genotype (LS2) and the same parameters as in the first experiment. Results indicated that 8 µM BAP was the best concentration to promote shoot node formation, with an average of 4.7 nodes per explants (Fig. 9–A). However, the highest BAP concentration used (10 µM BAP) showed to be more effective for shoot proliferation (Fig. 9– B) with an average of 1.7 shoots per explant whereas 2 µM BAP was better for shoot growth (Fig. 9–C) with shoots displaying an average length of 5.02 cm. Statistical differences were found between the control and 2, 8 and 10 µM BAP concentrations. No significant difference was found between 1and 4 µM BAP concentrations compared with and all the others.

A comparison between the different BAP concentrations tested is shown in Fig. 10 where can be seen that, for all the parameters analysed, results were higher than in the control. The morphological aspect of the cultures in the presence of the tested BAP concentrations can be seen in Figure 11.

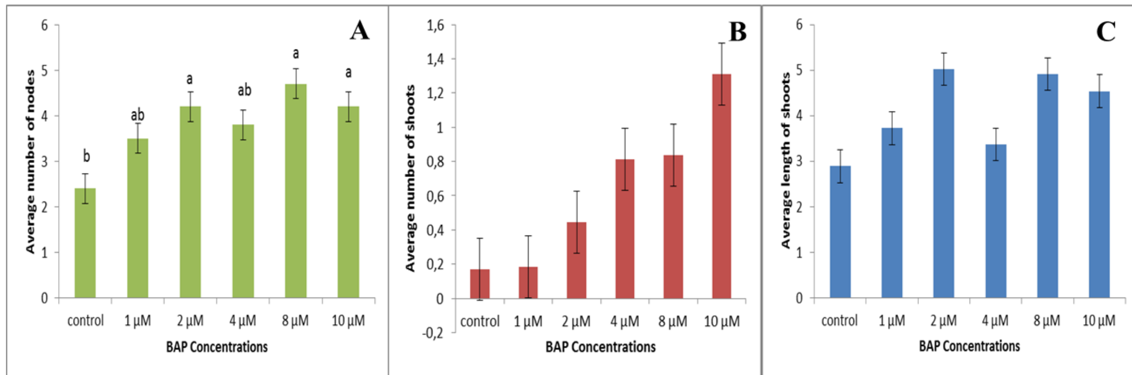

Figure 9 –Number of nodes (A), average number of shoots (B) average shoots length (C) in shoot tip culture of *L. Sativus* on a medium containing different concentration of BAP (1 µM, 2 µM, 4 µM, 8 µM and 10 µM). Values are the mean ± SE of three replicates and those followed by the same letter are not significantly different at the P<0,05 level using the Tukey test.

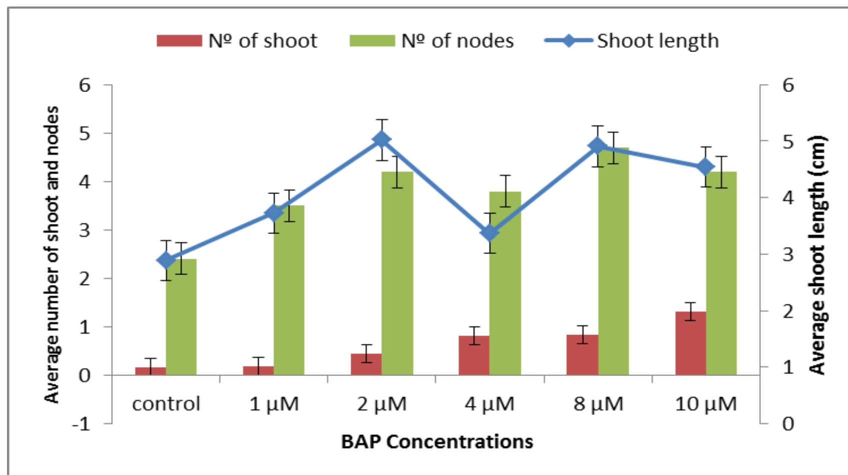

Figure 10 – Comparison between the response different medium BAP concentrations to shoot tip culture.

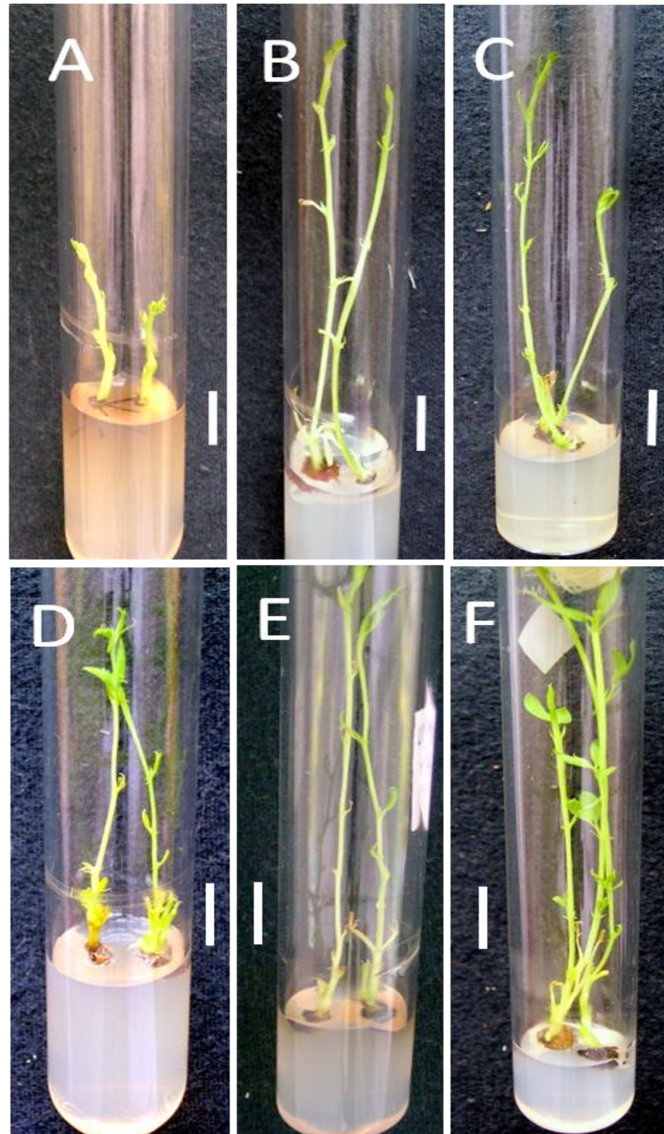

Figure 11- Shoot tip cultures of *L. sativus* on media containing different concentrations of BAP: (A) - Control, (B)- 1µM BAP, (C)- 2µM BAP, (D)- 4 µM BAP, (E)- 8 µM BAP and (F)- 10µM BAP. Pictures were taken after 5 weeks of culture. Bars =1 cm.

3.1.3 - *Lathyrus sativus* L. rooting.

Rooting assays were performed with shoots of the LS2 genotype developed on media containing 2 or 10 µM. Shoots were inoculated on a modified MS medium containing 2.46 µM IBA and compared with a control without IBA. The number of rooted shoots was very low with only one rooted shoot in the control medium and on the IBA medium. Due to the reduced number of roots produced, the effect of the shoot proliferation media (2 or 10 µM BAP) on rooting could not be evaluated. The figure 12 shows different stages of the rooting process.

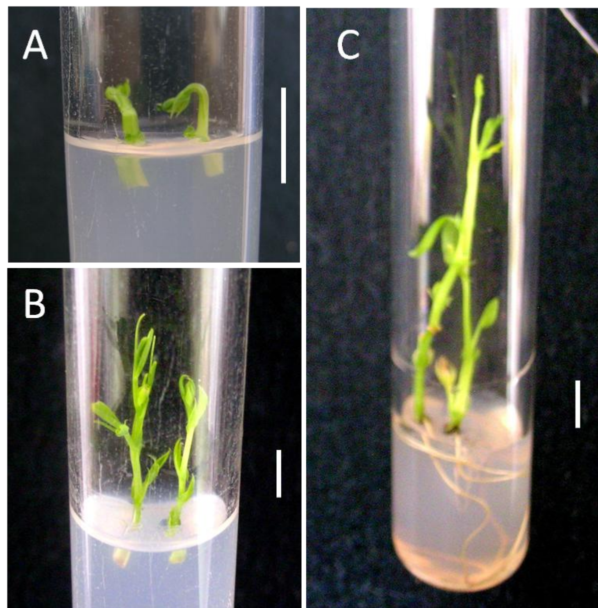

Figure 12 – Rooting of *L. sativus* shoots (A) Shoot tips inoculated on 2.46 µM IBA (B) Explant transferred to basal medium for root elongation two weeks later (C) Rooted plantlets

3.2- Diversity study of eight *Lathyrus sativus* L. Portuguese landraces.

3.2.1- Microsatellite diversity

The five polymorphic *L. cicera*-derived EST-SSRs detected a total of 13 different alleles in the 71 individual plants genotyped (Table VII). The number of alleles per locus ranged from two (*S037-022, S037-035* and *S132-100*) to four (*S037-011*) with

an average value of 2.6 allele per locus. PIC ranged from 0.139 (*S132-100*) to 0.567 (*S037-011*) with a mean value of 0.362. The most informative marker was *S037-011* with PIC (Polymorphic Information Content) value of 0.567.

Table VII- Allelic diversity of 5 microsatellite loci scored in eight *L. sativus* populations (n = 71)

No	SSR *locus*	Repeat Size	Size range (bp)	Na	PIC
1	*S037-011*	3	212-221	4	0.567
2	*S037-022*	3	113-116	2	0.335
3	*S037-035*	6	216-228	2	0.374
4	*S132-040*	3	273-288	3	0.395
5	*S132-100*	3	254-260	2	0.139
	Average			2.6	0.362
	Min			2	0.139
	Max			4	0.567

bp stands for base pairs

3.2.2- Diversity among populations

The average number for alleles detected per population was 2.225, being the lowest value (1.800) detected on populations LS4 and LS8 and the highest value (2.400) on populations LS1, LS2, LS5, LS6 and LS7 (Table VIII).

No private alleles per population were found.

The expected heterozygosity or gene diversity was on average 0.438, being the highest value (0.515) detected on LS7 population and the lowest (0.270) on LS4 population.

By performing Markov chain method in GENEPOP, and after adjusting for multiple tests with a sequential Bonferroni correction, three out of eight populations

showed significant ($p<0.01$) departures from Hardy-Weinberg equilibrium across loci, due to heterozygote deficiencies as we can see due to a positive inbreeding coefficient (F_{IS}) value in LS4 (F_{IS} 0.430), LS7 (F_{IS} 0.385) and LS8 (F_{IS} 0.540) (Table VIII). This means that there are more homozygous in those populations than it would be expected in a population in Hardy-Weinberg equilibrium.

Table VIII- Within-population diversity estimates in eight *L. sativus* populations (n = 71)

Population	*Nav*	*Nar*	*Ho*	*He*	*Fis*	*P-value*	*P-value (Bonferroni)*	
LS1	2.400	1.969	0.347	0.390	0.111	0.042	0.084	ns
LS2	2.400	2.105	0.317	0.499	0.364	0.016	0.062	ns
LS3	2.200	2.122	0.357	0.492	0.274	0.095	0.095	ns
LS4	1.800	1.651	0.154	0.270	0.430	0.002	0.010	**
LS5	2.400	2.206	0.318	0.436	0.270	0.012	0.060	ns
LS6	2.400	2.236	0.391	0.506	0.227	0.021	0.063	ns
LS7	2.400	2.288	0.317	0.515	0.385	0.001	0.009	***
LS8	1.800	1.779	0.184	0.399	0.540	0.001	0.006	***
Average	2.225	2.045	0.298	0.438	0.325			
Min	1.800	1.651	0.154	0.270	0.111			
Max	2.400	2.288	0.391	0.515	0.540			

*N_{av} – average number of alleles; N_{ar} - allelic richness; H_O - observed heterozygosity; H_E - expected heterozygosity; F_{IS} - inbreeding coefficient: "***" corresponds to significance at the 0.1% nominal level, "**" significance at the 1% nominal level, "*" significance at the 5% nominal level and "ns" depicts non-significant values (after stepwise Bonferroni correction).*

3.2.3- Diversity among regions

Comparing the values of N_{ar}, H_O, H_E and F_{IS} for Alvaiázere and Penela (Table IX) populations no significant differences between these two collecting sample areas were found.

Table IX- Differences in average values of N_{ar}, H_O, H_E, and F_{IS} between Alvaiázere and Penela *L. sativus* populations.

Group	No. of populations	N_{ar}	H_O	H_E	F_{IS}
Alvaiázere	4	1.994	0.305	0.402	0.241
Penela	4	2.094	0.269	0.438	0.386
P*		0.619	0.477	0.589	0.141

**P-values obtained after 1,000 permutations*

3.2.4- Pairwise F_{ST} values between *Lathyrus sativus* L. populations

All pairwise F_{ST} values were not significant (P < 0.05), indicated an unrestricted gene flow among populations except between population LS4/LS5 (F_{ST} 0.00179, *P-value* < 0.05) and LS4/LS6 (F_{ST} 0.00179, *P-value* < 0.05). And even these values are so close to zero that we can consider that there is no relevant differentiation between the eight populations analyzed. LS4 and LS6 are from Alvaiázere and LS5 is from Penela (Table X).

Table X- Pairwise F_{ST} values between *L. sativus* populations. Below the diagonal are their respective P-values for significant differences.

Population	LS1	LS2	LS3	LS4	LS5	LS6	LS7	LS8
LS1	0.000	0.250	0.023	0.009	0.289	0.257	0.729	0.007
LS2	ns	0.000	0.132	0.109	0.584	0.279	0.657	0.229
LS3	ns	ns	0.000	0.291	0.009	0.188	0.409	0.082
LS4	ns	ns	ns	0.000	0.002	0.002	0.039	0.184
LS5	ns	ns	ns	*	0.000	0.588	0.843	0.023
LS6	ns	ns	ns	*	ns	0.000	0.966	0.009
LS7	ns	ns	ns	ns	ns	ns	0.000	0.127
LS8	ns	ns	ns	ns	ns	ns	ns	0.000

"" significance at the 5% nominal level and "ns" depicts non-significant values*

3.2.5- Analysis of molecular variance (AMOVA)

Considering the result of the AMOVA (Table XI) most of the molecular variance detected is due to differences within populations (93.27 %) and only 6.73 % of the variance can be associated to differences among populations. These small differentiation detect by AMOVA translates to a small, but significant ϕ-value of 0.067. Because of this result we tested the partitioning of the variance between the populations from Alvaiázere *versus* those collected in Penela. This analysis show us that the small differentiation detected initially could not be explained by differences between Alvaiázere and Penela (the ϕ-value is no longer significant).

Table XI- AMOVA analysis for the partitioning of microsatellite diversity (1) among and within *L. sativus* populations, (2) between two different populations origin (Alvaiázere *vs.* Penela), among populations within different origins and within populations.

Analysis	Source of variation	df	Percentage of variation	ϕ-statistics	p(ϕ)
(1)	Among populations	7	6.73	$\phi_{ST} = 0.067$	< 0.01
	Within populations	134	93.27		
(2)	Between Alvaiázere and Penela	1	1.20	$\phi_{CT} = 0.012$	0.255
	Among populations within different origins	6	6.01	$\phi_{SC} = 0.061$	< 0.05
	Within populations	134	92.79	$\phi_{ST} = 0.072$	< 0.01

3.2.6- Factorial correspondence analysis (FCA)

Factorial correspondence analysis (FCA) generated by the appropriate resampling scheme of the dependent objects, in the case, alleles between individuals, gave the possibility to detecting structural relationships among variable categories.

The analyzed objects (groups of individuals) are visualized as a group of dots in a hyperspace that has as many dimensions as terms (alleles) for all of the variables.

The results of FCA for the eight populations show the genetic relatedness among the 71 genotypes of *L. sativus* in a 2-dimentional space, where in the first axis is represented 42.12 % of the total variance and in the second axis it is represent 26.93 % of the total variance. In this way, in this 2D plot we have represented 69.05 % of the total variance of the dataset. Even though there are no well-defined clusters, each population is very diverse in itself, as the dots spread across the space can show. However is it possible to observe that the LS3 population is positioned on the 1st quadrant of the graphic; LS5 is mainly on the 4th quadrant; LS1 consenter the majority

of the individuals on the 3th quadrant and on the 2th quadrant we can find the barycentres of LS8 and LS4 accompanied with a large number of the correspondent individuals (Figure 13).

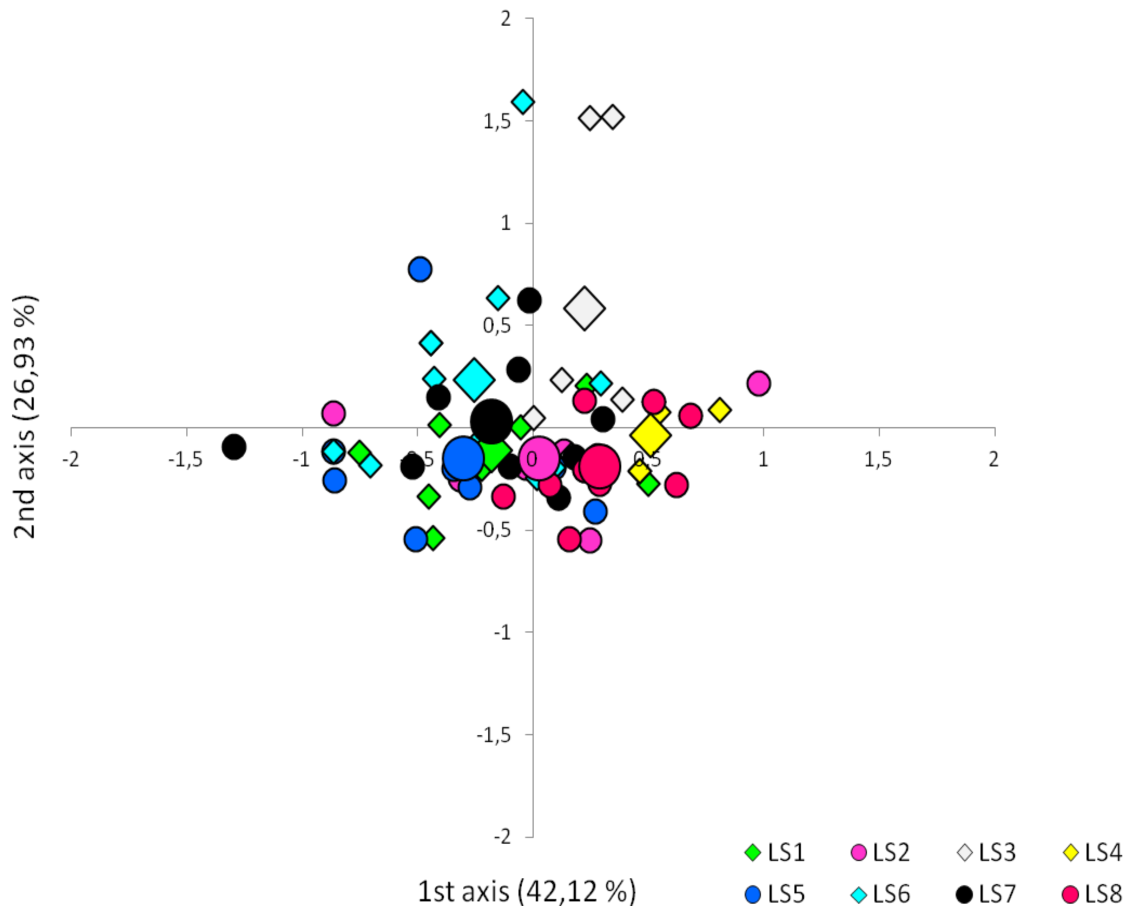

Figure 13- Factorial correspondence analysis (FCA) of 71 *L. sativus* plants belonging to eight populations. Each individual genotype is indicated by a small sign, while the population barycentres are represented by larger ones.

3.2.7- Neighbor-Joining tree

The resulting dendogram from the cluster analyzing using the Neighbor-Joining algorithm indicated that there are no well define and clear clusters, as showed by Factorial Correspondence Analysis (FCA) and the branches are not supported by bootstrap values (Figure 14). No correlations exist with the collecting geographical location. Even though only used 5 SSRs were used most of the individual could be distinguished from each other except for LS5I05/LS6I10 (from Penela and Alvaiázere respectively); LS5I22/LS6I08 (from Penela and Alvaiázere respectively); LS4I25/LS7I12 (from Alvaiázere and Penela respectively); LS8I09/LS8I13; LS4I03/LS4I20; LS7I04/LS1I02 (from Penela and Alvaiázere respectively); LS4I04/LS4I12/LS8I02 (from Alvaiázere and Penela respectively); LS4I16/LS8I12 and LS1I26/LS6I03(both from Alvaiázere) that are equals between them.

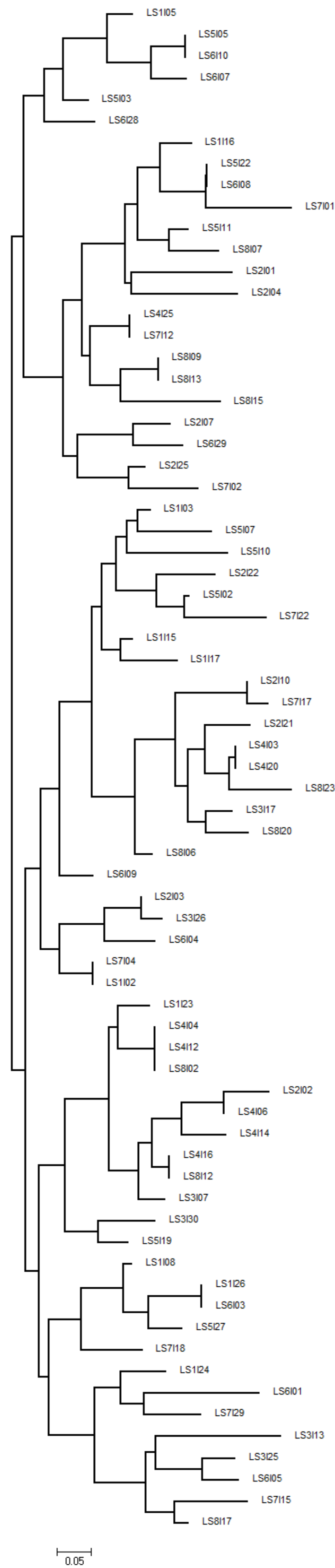

Figure 14- Neighbor-Joining tree based on the proportion of shared alleles distance between pairs of 71 *L.sativus* plants belonging to eight different Portuguese populations.

3.3-NMR analyses of β-ODAP and isomerisation thermal kinetics

3.3.1-^{1}H-NMR identification of β-ODAP

^{1}H-NMR spectroscopy can be used to distinguish between β-ODAP and α-ODAP isomers since their aliphatic protons are chemically and magnetically distinct, thus possessing distinct chemical shifts (δ, ppm) and as well distinct ^{1}H-^{1}H coupling constants (J). Figure shows the ^{1}H-NMR spectrum of β-ODAP. Two sets of resonances are observed; the first set (δ~4.10 ppm) due to the H_x proton (attached to the α-carbon), and consists of a duplet of duplets, while the other set is due to the H_a and H_b protons (attached to β-carbon), forming a typical ABX system.

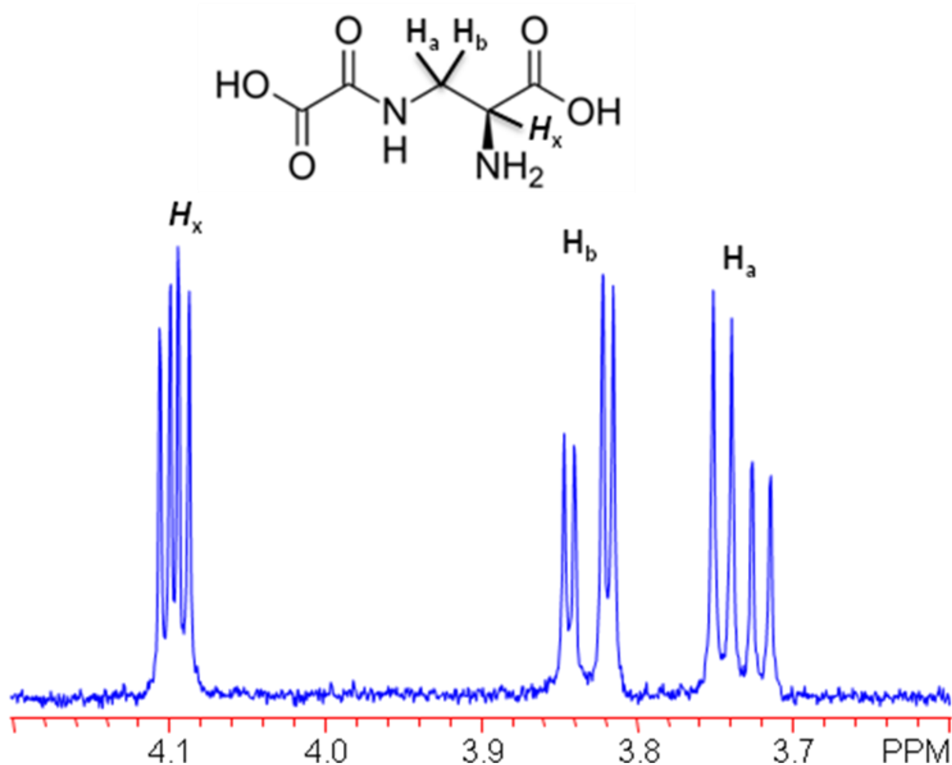

Figure 15-^{1}H NMR spectrum of β-ODAP and identification of aliphatic proton resonances.

The conversion between β-ODAP and α-ODAP can be achieved through an increment in temperature of the sample. The above sample was subject to a temperature of 75°C for some time (~1hr) and the resulting spectrum is presented in Figure 15. As can be seen in Figure 16 a new ABX system, due to the α-ODAP isomer, is present. These two isomers are thus very distinguishable in a ^{1}H-NMR spectrum and their

presence can in principle be determined in samples of seeds from *L. sativus* L. Portuguese landraces.

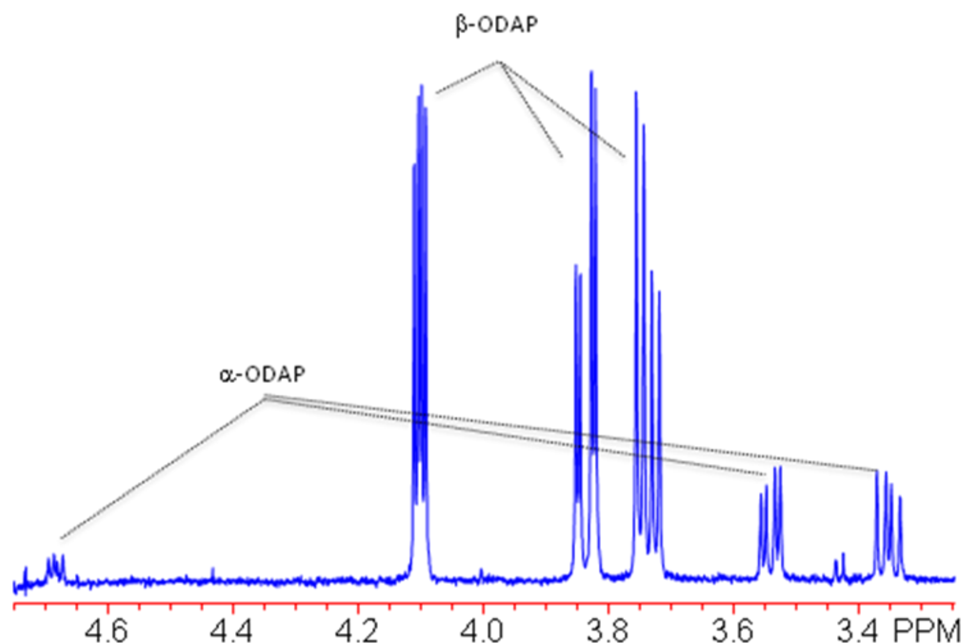

Figure 16-^{1}H NMR spectrum of a mixture of β-ODAP and α -ODAP, after exposure of solution at 75°C. Resonances due to the α-ODAP isomer are seen in periods of less than 1 hr.

3.3.2- Quantification of β-ODAP in eight *Lathyrus sativus* L. Portuguese landraces.

A ^{1}H-NMR analysis of the ethanol: H_2O extracts of eight *L. sativus* L. Portuguese landraces was made and the expansions of the regions from the NMR spectra containing the resonance due to the Hx proton are presented in figure 17. This allows a relative estimate of the content of β-ODAP by comparison with the resonance at 4.16 ppm, which remains essentially constant throughout all samples analyzed. An absolute quantification of the percental (% of dry weight) contribution of β-ODAP to whole composition of the seed by NMR requires the inclusion of an internal standard, which was not made in these assays. Nevertheless, a comparison between samples is possible by means of the normalization of the areas to the constant element (δ=4.06 ppm). As it can be appreciated sample LS4 is the one possessing the highest content of

β-ODAP, while LS3 possesses the lowest. All other samples are essentially identical in β-ODAP contents.

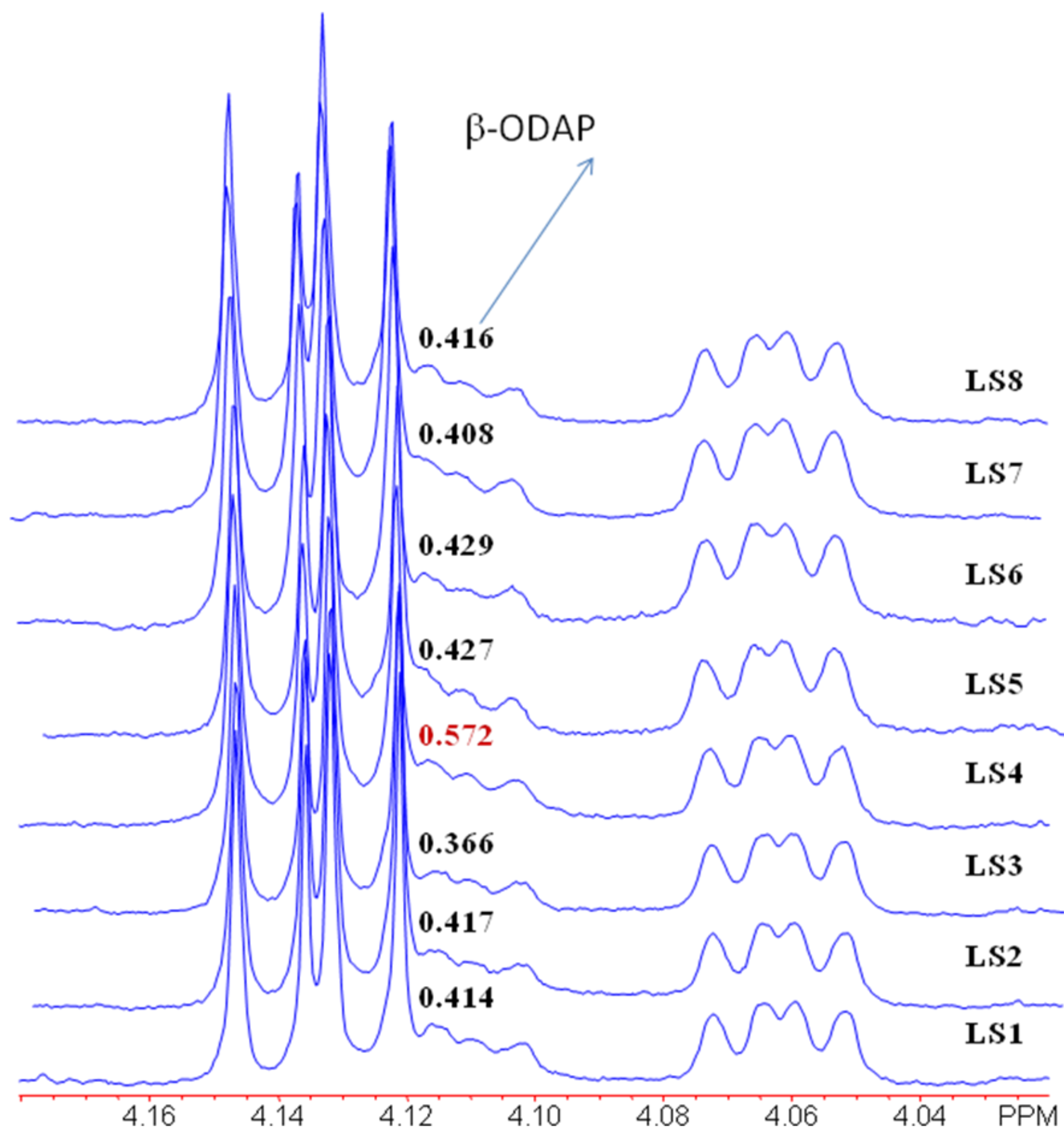

Figure 17-^{1}H-NMR spectra of samples LS1 to LS8. Numbers refer the relative content in β-ODAP in each sample by comparison to the resonance at δ=4.06 ppm, which remains constant in all samples.

3.3.3-Thermal kinetics of β-ODAP isomerisation

In order to determine the Kinetics of thermal isomerisation of β-ODAP into α-ODAP, 5 samples of synthetic formula of β-ODAP were prepared. One was not subject

to any thermal treatment but the other 4 were subject to boiling water (T ~100°C) for different time periods: 10, 20, 30 and 60 minutes. The same was made to 5 samples of LS4 ethanol: water extract. The choice of the boiling periods was made in order to estimate possible alterations in β-ODAP contents due to normal cooking procedure. The spectrum in figure 18 shows the temporal evolution in the contents of β-ODAP and α-ODAP upon submission to a temperature of ~100°C. This isomerisation shows time dependence and clearly indicates that by the end of the 60 minutes period it essentially reaches a steady state condition (only 2% increase in α-ODAP content in the last 30 minutes).

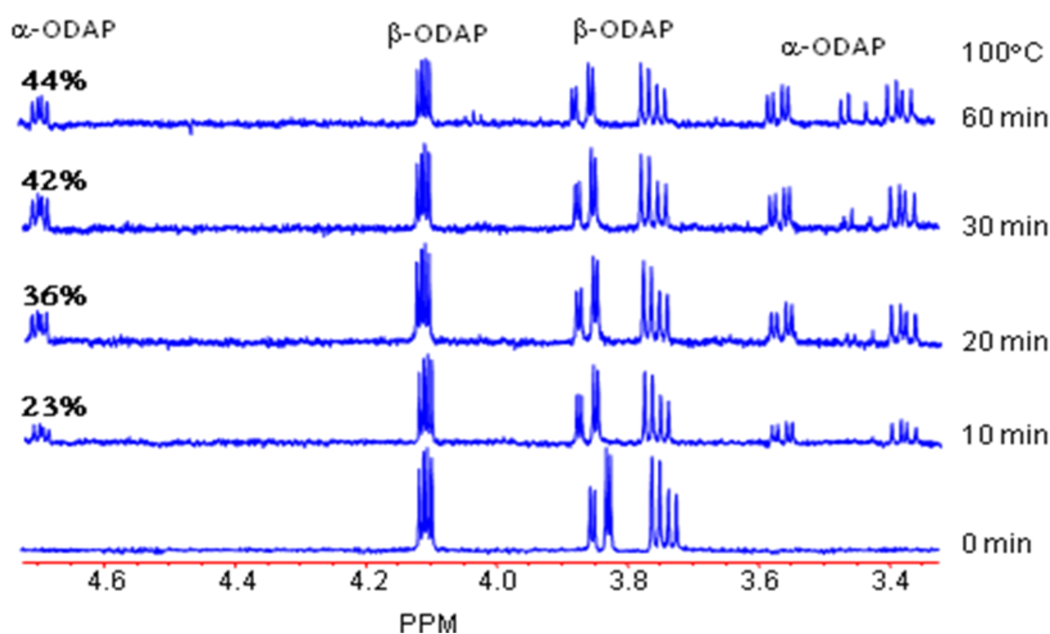

Figure 18-^{1}H NMR spectra of samples of β-ODAP subject to different periods at 100°C. With time the content in α-ODAP increases, denoting a thermal isomerisation phenomenon, which is fast at this temperature.

The same thermal analysis was made in LS4 samples and the expansions of figure 19 show the reduction in β-ODAP contents in LS4 samples by exposure to 100°C.

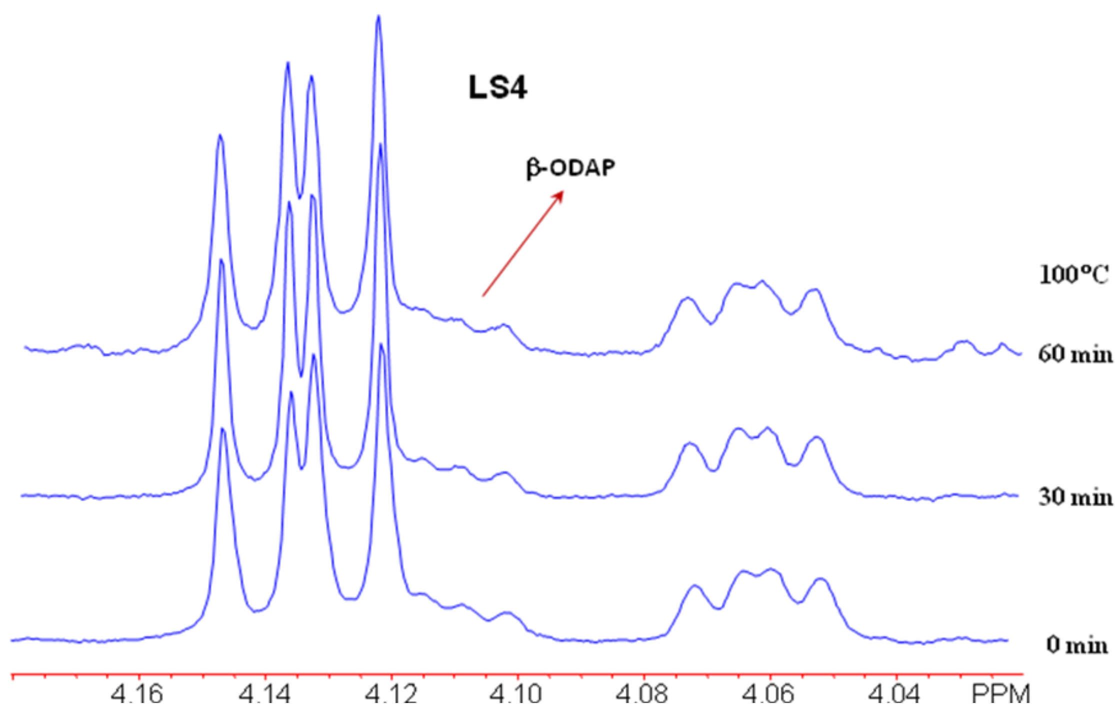

Figure 19-^{1}H-NMR spectra of LS4 samples subject to different periods at 100°C. With time the content in β-ODAP is reduced.

To demonstrate that the thermal conversion in LS4 samples occurs by means of formation of α-ODAP, Figure 20 illustrates the appearance of α-ODAP as a result of exposition to boiling water. The major difficulty resides in the minute amounts of α-ODAP and the need for solvent pre-saturation. Since the resonance due to residual solvent (HOD) is very near that of α-ODAP at 25°C, some saturation also occurs for the later, making it more difficult to be observed.

The results demonstrate that β-ODAP is the only isomer present in the samples of *L. sativus* L. Portuguese landraces. The conversion of this isomer into the α-ODAP requires thermal activation. An exposition to 100°C for several minutes is sufficient to promote a significant interconversion, which remains stable upon temperature reduction. Boiling of the seed or seed powder is able to reduce the levels of β-ODAP to almost 56% by the end of 60 minutes, but significant amounts of the β-ODAP isomer still remain, suggesting that this procedure by itself is unable to achieve desirable reductions of β-ODAP contents.

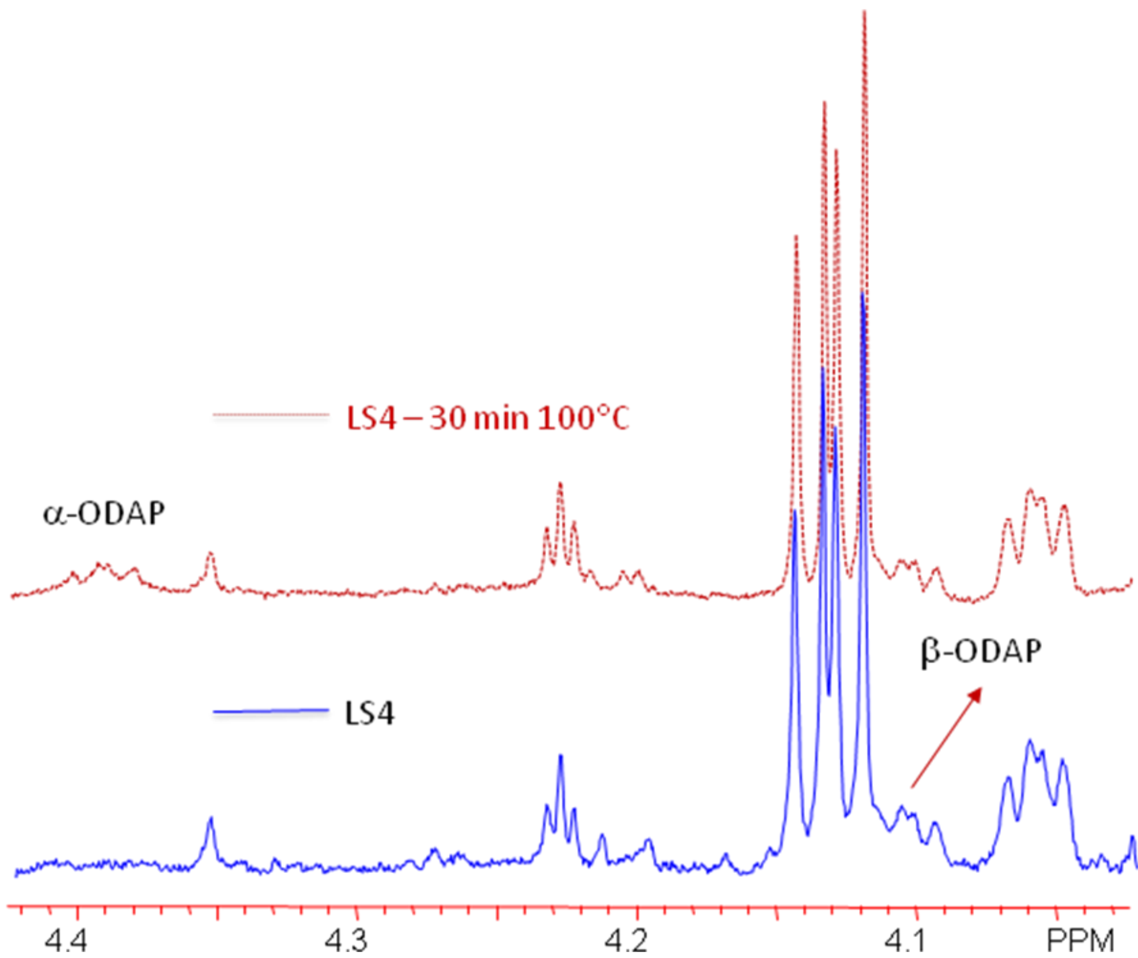

Figure 20-^{1}H-NMR spectra of LS4 samples: no boiling (bottom spectrum) and boiling for 30 min at 100°C (top spectrum). With time the content in β-ODAP is reduced and α-ODAP builds up.

4. DISCUSSION

4.1–Micropropagation of *Lathyrus sativus* L. Portuguese landraces.

Micropropagation through shoot proliferation and further rooting was observed in a large number of species (Canhoto 2010), including several members of the Fabaceae family (Kendil *et al.,* 2009). In this work, the ability of 8 landraces from Portugal were tested. The results showed that micropropagation of *L. sativus* can be achieved through the culture of shoot tips obtained from germinated seedlings. No significant differences could be found among the genotypes tested. The genotype of the explants is a key factor for micropropagation of some species since it is usually observed that different genotypes have distinct behaviors when tested *in vitro* (Ochatt *et al*., 2004). These authors found different responses for shoot regeneration on genotype and hormonal combination specific for each genotype. The results in *L. sativus* may be explained by the fact that the explants used in the *in vitro* assays were from young seedlings germinated *in vitro*. Shoot proliferation from seedlings does not assure the propagation of a selected genotype since the quality of the plant cannot be determined at this stage. However, these preliminary results may be important to establish the ideal *in vitro* conditions to propagate selected plants that can show a particular characteristic of interest such as a better seed production or an increased tolerance towards biotic or abiotic factors.

Cytokinins are usually used to promote axillary shoot proliferation or shoot tip growth (Canhoto, 2010). In this work, the effect of a wide range of BAP concentrations on *L. sativus* shoot tip culture was analysed. The results showed that for all the parameters recorded the presence of BAP, at any of the tested concentrations, improved the response over the control. The main objective of micropropagation through axillary shoot proliferation is to produce, in each subculture, a considerable amount of new meristems that can be stimulated to growth again to repeat the process until the desired amount of plants can be achieved. The highest number of nodes per explants, in our assays, was 4.7 on a medium containing 2µM BAP. Although further experiments were not carried out, this means that in each subculture, the number of expected plants produced will be multiplied by a factor near 5 which can be considered an interesting rate of shoot propagation. However, it must be stressed out that this rate was obtained with seedling explants and that the same type of experiments could give a considerable lower multiplication rate whether explants from adult plants had been used. Barik *et al.*

(2005) used different explants types such as, cotyledons, hypocotyls, epicotyls, internodes and leaves, for plant regeneration. The explants were inoculated on a MS basal medium supplemented with the growth regulators BAP (2.22 – 2.19 μM) and α−naphthalene acetic acid (NAA, 2.69 – 16.11 μM alone or in combinations. A direct comparison between Barik *et al*. (2005) experiments and our work cannot be made since the authors pretended to evaluate the regeneration response of different explants in different culture medium. However, the organogenic type of regeneration found by these authors can be an alternative to our protocol and deserves to be tested in the Portuguese genotypes.

Rooting is a crucial step for the success of any micropropagation protocol. High rates of shoot proliferation are worthless whether rooting cannot be achieved. Several authors, using different culture conditions were able to promote root *in vitro* root formation in *L. sativus*. Thus, Ochatt *et al*. (2004) obtained the best rooting responses on a half-strength hormone-free MS medium, but differences were apparent between regenerated shoots depending on the hormonal balance used for the induction of regeneration from explants. Barik *et al.* (2005) obtained the best result with maximum root number and longest average root length with IAA at 2.85 μM. However, no root formation was observed when shoots were cultured in the medium devoid of auxins. Nevertheless, roots were able to elongate only after the rooted shoots were transferred to half-strength MS lacking the auxin. In general, auxins are known to promote root induction, but they inhibit subsequent root growth if allowed to persist in the culture medium (Barik *et al*., 2005). The experiments carried out with the Portuguese genotypes showed that, in the tested conditions, rooting was only sporadically induced. This means a serious constraint to *L. sativus* micropropagation. Further studies must be carried out testing different conditions (IBA concentrations, other auxins…) in order to improve the rooting levels.

4.2- Genetic diversity of *Lathyrus sativus* L. Portuguese landraces.

Although grass pea is generally consider a marginal crop, almost in extinction (Lioi *et al*., 2011), grass pea landraces are still maintained on farm in Portugal, mainly in dryland farm systems in the poor soils of limestone areas, such as is Alvariázere and Penela. Today, grass pea in not included in the official statistics of grain production in Portugal. However there is a renewed interest in grass pea due to the agricultural features, such as drought tolerance, nitrogen fixation, tolerance to salinity and low soil fertility and the ability for resist to the natural disasters associated to climate changes. Grass pea can provide human and animal feed, under condition unsuitable for economic production of other legume crops (Hillocks & Maruthi, 2012).

To our knowledge this is the first diversity study made with Portuguese *L. sativus* landraces using EST-SSRs. Sardinha *et al*., (2007) and Almeida *et al*., (2009) studied the diversity in an Iberian collection (including some accessories from Algarve) using AFLPs, however the studies did not consider populations and only one accession was used. In a study by Belaid *et al*. (2006), Portuguese *L. cicera* genotypes were tested to verify the genetic diversity and quantify the intra-specific variation of populations form different geographical regions.

In the present study *L. cicera* EST-SSRs derived-markers were used as a tool for assessing genetic variation and determining the relationships among eight different populations of *L. sativus* with two different origins (Alvaiázere and Penela). The average PIC value obtained was 0.362. That suggests a moderate level of polymorphism of the *L. cicera* EST-SSRs derived-markers. Shiferaw *et al.* (2012) using germplasm from different regions of Ethiopia, made a study using 11 EST-SSR developed from *L. sativus* and EST-SSR transferable from *M. truncatula* and obtained an average PIC value of 0.416. Lioi *et al.* (2011) used EST- SSRs *L. sativus* and *L. japonicus* derived-markers to study the diversity in Italian grass pea. As the EST-SSRs origin, from different donor species, and the statistic used for the evaluation of the EST-SSRs informativeness was based on Nei´s formula, different from our study, make a direct comparison very difficult. Nevertheless all the three studies have confirmed the value of EST-SSRs in assessing grass pea diversity. Our data provide evidence of a moderate genetic diversity within population and a very low genetic differentiation among

populations. The total gene diversity across all accessions (mean 0.438) in our study, with landraces, was similar then the reported by Shiferaw *et al*. (2012) (mean 0.419), using Ethiopian germplasm provided by the Institute of Biodiversity Conservation (20 accessions and 12 individual per accession). For both, LS4 and LS7, the higher intra-population variation seems to have no relation with the geographical origin.

Nevertheless, although the studied landraces present a high genetic diversity levels, reflected on the expected heterozygosity values (0.438), gene diversity is defined as the probability that two randomly chosen alleles from the population are different (Upadhyaya *et al.*2008), no clear clustering based on genetic distance was observed among the analyzed populations. No private alleles per population were found, and by the pairwise test for genetic differentiation only between three populations (LS4/LS5 and LS4/LS6) the results were significant. This may have been due the gene flow among populations, such as the seed exchanged because the two locations are near.

In the case of the origin assessment we did not detect significative genetic diversity between these two collecting sample areas. This may be explained by the proximity of these two areas, the distance between the two locations of collection (Alvaiázere and Penela) is around 25 Km, so they are quite near. Besides that, Alvaiázere and Penela are both rural areas with several agricultural markets, a meeting point for goods exchange, each means a great possibility for farmers to buy and sell their own grass pea seeds to other farmers. Usually, farmers select their own seeds for sowing but some of them explained that they already bought from other local farmers. The obtained results are not surprising regard the oversaid. This study could be more interesting if we had analysed landraces from other regions, but we had a restrict time to perform the study and this was the near location from us.

The AMOVA analysis revealed that the high diversity detected is justified by the existing diversity within populations (93.27%).In contrast there is no differentiation based on the origin assessment. *L. sativus* is mainly self-pollinator specie, however the AMOVA results doesn´t match with that concept. This is a not expected result since *L. sativus* is mainly autogamous. Kumar *et al.* (2011) observed, in the Mediterranean environment, 20-27% out-crossing depending on the local conditions.

The deviations from the Hardy-Weinberg equilibrium verified in LS4 population may be explained based on the fact that this population is hold by a marked oriented farmer, which make selection for large and white grain, the most desirable for the European markets (Ulloa & Mera, 2010). The Hardy-Weinberg equilibrium states genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences; therefore, the selection procedure made by man influences the evolutionary process.

Population genetic structure across the accessions analyzed didn't identified any specific groups; the genetic distances between all accessions were very narrow. The gene flow due to the movement of seeds can explain the low genetic differentiation. Seed exchange among farmers is a usual procedure to enhance diversity of local germplasm which may result in an increase in the distribution of alleles among different populations irrespective of their geographical distance (as resumed by Shiferaw, 2012).

This study revealed some limitations, such as a restricted collected landraces area. It would be interesting, for the future, to study the genetic diversity among landraces from other Portuguese regions, such as Algarve and Sintra, and compare with grass pea landraces from other countries. It is possible that the restricted number of SSRs used may influence the results although because EST-SSRs usually are polymorphic and can present high value of PIC as Almeida *et al.* (2013) demonstrated on his work, we considered that this number could be enough to study the genetic diversity proposed. However, in future assays it would be interesting to address a larger number.

The unexpected result for the high diversity within populations, more probable in an out-cross pollinator species that in a self pollinator, makes the study of the cross pollination rates of these landraces very interesting to the future.

4.3- NMR analyses of β-ODAP and isomerisation thermal kinetics

^{1}H-NMR spectroscopy can be considered an interesting and reliably method to identify and distinguish between β-ODAP and α-ODAP isomers. Based on a purified β-ODAP sample the identification of β-ODAP in the eight landraces studied was carried

out the values measured are a relative estimation of the content of β-ODAP by comparison with the resonance at 4.16 ppm, which remains essentially constant throughout all samples. It will be interesting, in the future, to address an absolute quantification of the percentual (% of dry weight) contribution of β-ODAP to whole composition of the seed, which allow a comparison with published quantifications value. Nevertheless, a comparison between samples is possible therefore sample LS4 is the one possessing the highest content of β-ODAP, while LS3 possesses the lowest. All other samples are essentially identical inβ-ODAP content. Is important to know these measures to have a more accurate idea about the grass pea potential toxicity once the β-ODAP is the compound responsible for the nocive effects expressed in humans and animals as a consequence of overconsumption (Lambein, 2007).

Exposition of β-ODAP to high temperature using boiling water promoted the isomerisation of β-ODAP into α-ODAP. The isomerisation was dependent o the exposure time and after an exposition period of an hour, a steady was reached in which the levels of β-ODAP were reduced to 56% of the initial concentration. From these results it can be extrapolated that cooking it is an effective way to reduce the levels of this compound thus making the cooked seeds harmless for consumers. By the exposition to temperature using boiling water the isomerisation of β-ODAP into α-ODAP occurred. The isomerisation depends on time exposure but, by the end of the 60 minutes period it essentially reaches a steady state and reduces the levels of β-ODAP to almost 56%. Cooking the seeds it seems to be a process than can lower the β-ODAP content and increase the possibility in achieve a save *L. sativus* food.

5. CONCLUSIONS AND FUTURE PROSPECTS

5-Conclusions and future prospects

The final remarks on the investigation of these eight Portuguese landraces of *L. sativus* are:

I- A protocol for *L. sativus* shoot proliferation was achieved through seedlings shoot tip culture. Root formation was only sporadically induced and further studies are required to establish an effective micropropagation protocol for this species.

II- Genetic diversity and population structure were studied using *L. cicera* EST-SSRs microsatellites. A high degree of genetic diversity was found among individuals. However, when populations of the two localities were compared their genetic diversity was not relevant. Future work should involve populations from other localities in Portugal and abroad.

III- NMR allowed the detection and relative quantification of β-ODAP in the different populations, showing minor differences between them. The kinetic conversion of β-ODAP into α-ODAP was also monitored by this technique. It would be interesting to evaluate the levels of β-ODAP in processed food in order to determine the dietary intake of this compound by humans.

6. REFERENCE

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