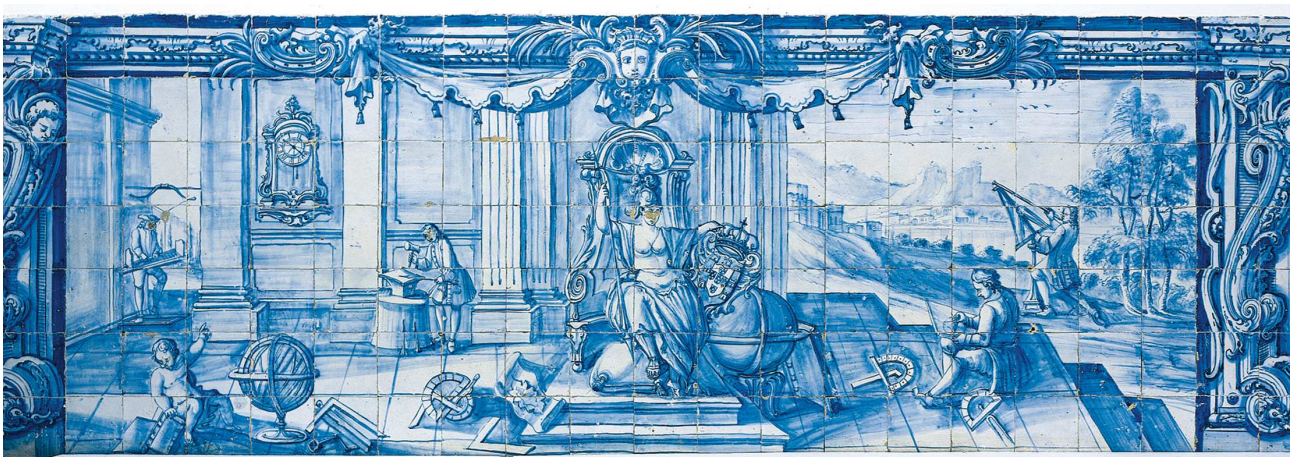
Characterization and evaluation of Portuguese *Opuntia* spp. germplasm

Carlos Manuel Gaspar dos Reis

Thesis presented to obtain the PhD degree
in Biology by the University of Évora

SUPERVISORS: *Professor Luiz Carlos Gazarini*
Professor Maria Margarida Ribeiro

ÉVORA, APRIL 2018



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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To my beloved parents

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Thesis publications

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Reis, C.M.G., Gazarini, L.C., Fonseca, T.F. & Ribeiro, M.M. (2018). Above-ground biomass estimation of *Opuntia ficus-indica* (L.) Mill. for forage crop in a Mediterranean environment by using non-destructive methods. *Experimental Agriculture*, 54(2), 227-242. https://doi.org/10.1017/S0014479716000211.

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Rodrigues, A.M., Pitacas, I.S., **Reis, C.M.G**. & Blasco-Ruiz, M. (2016). Nutritional value of *Opuntia ficus-indica* cladodes from Portuguese ecotypes. *Bulgarian Journal of Agricultural Science*, 22(1), 40-45. http://www.agrojournal.org/22/01-07.pdf.

Reis, C.M.G., Gazarini, L.C. & Ribeiro, M.M. Fruit production from *Opuntia ficus-indica* ecotypes in comparison to commercial Italian clones. *Horticultural Science* (in press).

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Reis, C. M. G., Pitacas, F. I. & Rodrigues, A. M. (2017). Estudo do valor nutricional dos cladódios de ecótipos de figueira-da-índia (*Opuntia ficus-indica*). *XXXVIII Reunião de Primavera da Sociedade Portuguesa de Pastagens e Forragens. Escola Superior Agrária de Castelo Branco, Castelo Branco, Portugal, 27 e 28 de Abril* (poster).

Reis, C.M.G, Gouveia, C., Vitorino C., Gazarini, L.C., Ribeiro, M.M. & Peres, F. (2016). Ácido ascórbico, betalaínas, e fenóis totais em ecótipos de *Opuntia* spp*. Atas do XIII Encontro de Química dos Alimentos, Porto, Portugal, 14-16 September, pp. 35-38* (oral communication).

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ambiente e agricultura, Universidade de Évora, Évora, Portugal, 20-21 October (oral communication).

Reis, C.M.G, Gazarin, L.C. & Ribeiro, M.M. (2015). Avaliação de ecótipos de figueira-da-índia (*Opuntia ficus-indica* (L.) Miller) para produção de fruto. *Congresso Nacional das Escolas Superiores Agrárias, Escola Superior Agrária de Bragança, Bragança, Portugal, 2-3 December* (poster).

Reis, C.M.G, Gazarin, L.C. & Ribeiro, M.M. (2015). Biometria do fruto da figueira-da-índia [(*Opuntia ficus-indica* (L.) Miller]. *III Jornadas do Potencial Técnico e Científico do IPCB, Escola Superior Agrária de Castelo Branco, Castelo Branco, Portugal, 25 November* (oral communication).

Reis, C.M.G, Gazarini, L.C. & Ribeiro, M.M. (2015). Portuguese ecotypes of *Opuntia ficus-indica* differ in biomass production. *Jornadas Portuguesas de Genética, Universidade do Minho, Braga, Portugal, 25-27 May* (poster).

Reis, C.M.G., Pitacas, F.I. & Rodrigues; A.M. (2014). Produção de biomassa e análise do teor de proteína em populações portuguesas de *Opuntia ficus-indica* (L.) Mill. *1º Encontro Nacional do figo-da-índia, Évora, Portugal, 12 April* (oral communication)

Reis, **C.M.G**., Ribeiro, M.M. & Moreira, L. (2013). Estimating biomass production in Portuguese germplasm of *Opuntia ficus-indica* (L.) Miller. *VIII International Congress on Cactus Pear and Cochineal, University of Palermo, Italy, October 28-31* (poster).

Characterization and evaluation of Portuguese *Opuntia* spp. germplasm

Abstract

The main objectives of this thesis were to characterize and evaluate Portuguese *Opuntia* spp. ecotypes for biomass production and the cladodes nutritional quality for fodder, and for fruit yield and quality. In addition, the genetic diversity was assessed with nuclear microsatellite (nuSSR) markers. The plant vigour and biomass production were evaluated in germplasm of *O. ficus-indica* by non-destructive methods, 3 years following planting. Among ecotypes, significant differences were found in the studied biomass-related parameters and several homogeneous groups were established. In the case of the cladodes nutritional profile significant, differences were found in the crude protein and the ash content, and different groups were unfolded. In general, *O. ficus-indica* has a low dry matter content, crude protein, and neutral detergent fiber, and high content in non-fiber carbohydrates and metabolizable energy. Fruit production was evaluated in the second and third years after plantation. Significant differences were found among *O. ficus-indica* ecotypes, and different groups were established. The Italian cultivars “Gialla” and “Bianca” had highest fruit yield than Portuguese ecotypes. Besides, the morphology, bioactive compounds and antioxidant properties of fruits were studied in twenty ecotypes belonging to the species *O. ficus-indica*, *O. robusta*, *O. dillenii* and *O. elata*. The fruits displayed variability in morphological and bioactive characteristics. The analysis of genetic diversity using nuSSR markers within a set of 19 ecotypes, belonging to the four previously-mentioned species, was undertaken. The hierarchical clustering analysis revealed four major groups that clearly disentangled the *Opuntia* spp. species. Two subclusters were found considering the *O. ficus-indica* ecotypes. The results revealed a low level of genetic diversity among the ecotypes of *O. ficus-indica.*

Caracterização e avaliação de germoplasma Português de *Opuntia* spp.

Resumo

A presente tese teve como principais objetivos a caracterização e avaliação de ecótipos Portugueses de *Opuntia* spp. no que se refere à produção de biomassa, qualidade nutricional dos cladódios como forragem e produção e qualidade do fruto. Adicionalmente, foi avaliada a sua diversidade genética por microssatélites nucleares. A produção de biomassa em ecótipos de *O. ficus-indica* foi avaliada por métodos não destrutivos nos primeiros três anos após a plantação. Foram encontradas diferenças significativas nos parâmetros relacionados com a produção de biomassa e vários grupos homogéneos foram estabelecidos. No caso do perfil nutricional dos cladódios, foram encontradas diferenças significativas para a proteína bruta e teor de cinzas. Em geral, *O. ficus-indica* tem baixo teor de matéria seca, proteína bruta e fibra em detergente neutro e alto teor de hidratos de carbono não fibrosos e energia metabolizável. A produção de fruto em *O. ficus-indica* foi avaliada no segundo e terceiro anos após a plantação. Diferenças significativas foram encontradas entre ecótipos e diferentes grupos foram estabelecidos. As cultivares italianas "Gialla" e "Bianca" apresentaram maior produção de fruto comparativamente aos ecótipos portugueses. Em 20 ecótipos pertencentes às espécies *O. ficus-indica*, *O. robusta*, *O. dillenii* e O*. elata*, foi estudada a morfologia do fruto, a composição em ácido ascórbico, betalaínas e fenóis totais, bem como a atividade antioxidante. Os frutos mostraram variabilidade nas várias características estudadas. Foi realizada a análise da diversidade genética, em 19 ecótipos pertencentes às quatro espécies anteriormente mencionadas, com utilização de microssatélites nucleares. A análise de agrupamento hierárquico revelou quatro grandes grupos que separaram claramente as quatro espécies de *Opuntia* spp. Entre os ecótipos de *O. ficus-indica* dois subgrupos foram constituídos. Os resultados revelaram um baixo nível de diversidade genética entre ecótipos de *O. ficus-indica*.

Abbreviations

°C – Degree Celsius
µL – Microlitre
µm - Micrometer
µM – Micromolar
6-FAM - 6-fluorescein amidite
A – Absorption value
AA – Ascorbic acid
ADF - Acid detergent fiber
ADL - Acid detergent lignin
AFLP – Amplified fragment length polymorphism
AMOVA - Analysis of molecular variance
ANOVA – Analysis of variance
AOAC – Association of Official Analytical Chemists
BC – Betalain content
bp – Base pair
CA – Cladode area
CAM – Crassulacean acid metabolism
CAp – Cladode area per plant
cm – Centimeter
CNp – Cladode number per plant
CP - Crude protein
CTAB - Hexadecyltrimethylammonium bromide
cv. - Cultivar
D – Diameter of the cladode neck
DAP – Days after plantation
DCPI - 2,6-dichloroindophenol sodium
DF – Dilution factor
DM - Total dry matter
DNA – Deoxyribonucleic Acid
DPPH - 1,1-diphenyl-2-picrylhyydrazyl
DW – Dry weight
DWp – Dry weight per plant
EE - Ether extract
f. – Form
FNp – Number of fruits per plant
FPp – Fruit production per plant
FW – Fresh weight
FWp – Fresh weight per plant
g – gram
GAE – Gallic acid equivalents

ha - Hectare
kg - Kilogram
L – Cladode length
ME - Metabolizable energy
Mg – Megagram
mg – Miligram
MI – Marker index
min - Minute
mL – Millilitre
mM – Millimolar
MW – Molecular weight
Na – Number of alleles
NDF - Neutral detergent fiber
NFC – Non-fiber carbohydrates
ng – Nanogram
nm - Nanometer
nuSSR – Nuclear microsatellites
OFI – *Opuntia ficus-indica*
PCA – Principal component analysis
PCoA – Principal coordinate analysis
PCR – Polymerase chain reaction
PIC – Polymorphic information content
r – Correlation coefficient
R^2 – Determination coefficient
RMSE – Root mean square error
rpm – Rotations per minute
s - second
SDS – Sodium dodecyl sulfate
spp. – Species
SSR – Simple sequence repeats or microsatellites
T – Cladode mean thickness
TDN - Total digestible nutrients
TE - Tris-EDTA
TPC – Total phenolic compound
TSS - Total soluble solids
U - Unit
UPGMA - Unweighted pair group arithmetic mean method
UV – Ultraviolet
V - Volt
W – Cladode width

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“Flor de cacto”

Flor de cacto, flor que se arrancou
À secura do chão.
Era aí o deserto, a pedra dura,
A sede e a solidão.
Sobre a palma de espinhos, triunfante,
Flor, ou coração?

José Saramago

1. An overview on *Opuntia* spp.

1.1 Origin, uses, and geographic distribution

In the Cactaceae family, the *Opuntia* spp. is a major recognized genus of the Opuntioideae subfamily, and the *Opuntia ficus-indica* (L.) Miller is the cactus species with the highest economic importance worldwide. The center of domestication for this species is localized in central Mexico (Griffith, 2004).

Several authors refered that cactus pear cacti were brought to the Iberian Peninsula by the first Spanish conquerors, between the end of the 15th century and the beginning of the 16th century (Barbera, *et al.*, 1992; Casas and Barbera, 2002), and, afterwards, spread all over the Mediterranean Basin (Le Houérou, 1996).

Today, these plants grow wild or are cultivated in the Southern Iberian Peninsula and around the Mediterranean region, in France, Greece, Israel, Italy and Turkey. The Arabs took them from Southern Spain to Northern Africa, where the species can be found in Algeria, Egypt, Eritrea, Ethiopia, Libya, Morocco and Tunisia. *Opuntia* spp. plants are widely distributed throughout the Americas from Canada to Chile: the Southern United States; all Central American and Caribbean countries; and the South American countries of Argentina, Bolivia, Brazil, Colombia, Peru and Venezuela. Wild and cultivated species of *Opuntia* spp. also grow in Angola, Australia, India and South Africa (Sáenz, 2013).

The most striking characteristics of *Opuntia* spp. are the anatomy and the morphology, which have enabled its adaptation to many highly stressful growing conditions, meaning that the plant is a viable option in regions where other plants will not survive (Sáenz, 2013), particularly in global change scenario. In addition, the species has a multiplicity of uses in agro-industry.

Opuntia ficus-indica (OFI) is grown for its large sweet fruits, which are interesting sources of functional compounds (Angulo-Bejarano *et al.*, 2014). The plant represents an interesting crop for cattle and sheep feeding by providing energy, water, and minerals during periods when food and water are scarce (Andrade-Montemayor *et al.*, 2011). The *Opuntia ficus-indica* is also used to produce young cactus pads consumed as a green vegetable, called “nopalitos” in Mexico (Sáenz, 2000), cochineal dye (Anderson, 2001) and as a medicinal plant (Kaur, 2012; Lim, 2012). The fruit's betalains could be used as natural colourants in food products (Gengatharan *et al.*, 2015); the oil from the seed could be used in the food, pharmaceutical and cosmetic industries (Ramadan and Mörsel, 2003; De Wit *et al.*, 2016); and the cladodes' physicochemical characteristics are suitable for biogas production (Jigar *et al.*, 2011). Cacti can also play a key role in erosion control and land rehabilitation, particularly in arid and semi-arid zones, and as a shelter, refuge and feed resource for wildlife (Le Houérou, 1996). Additionally, the cactus pear could be considered an option as

a carbon sink, absorbing and holding excess CO_2 in areas where the plant can be established, where nothing else can grow (Nobel and Bobich, 2002).

Although the species is widely dispersed, there are few official statistics, and information on planted areas and their use (e.g., fruit, vegetable, forage and cochineal breeding) is either not available or tends to be unreliable (Sáenz, 2013).

Concerning fruit production, available data indicate that the main producing country is Mexico, with a production of over 509 500 Mg fresh mass $year^{-1}$ on approximately 74 500 ha of specialized plantations (Flores-Valdez, 2010). Other countries that extensively cultivate the fruits include Italy, Chile, South Africa, Argentina, and Israel. In Europe, Italy has the most specialized cactus pear industry for fruit production. In this country, the cactus pear production is concentrated in Sicily, where cactus pear accounts for over 96.0% of the total Italian harvest with a surface area near 7 800 ha yielding approximately 80 000 Mg annually (ISTAT, mean data for the period 2009 - 2014).

In northern Africa, a cultivated area of 300 000 ha is estimated in Tunisia (Le Houérou 1996), 50 000 ha in Morocco (Arba *et al.*, 2002) including a large number of defensive hedges and non-specialized or semi-natural groves, 3 550 ha in Algeria (Inglese *et al.*, 1995), and 2 000 ha of semi-specialized plantations in Egypt (Bastawros, 1994).

Mexico is, essentially, the only country with *O. ficus-indica* commercial vegetable production, with “nopalitos” grown on approximately 13 500 ha (Flores-Valdez, 2010).

In terms of land area use, the greatest cultivation of *O. ficus-indica* is for forage or fodder, especially in Tunisia (500 000 ha), Brazil, and Mexico (Nefzaoui and Ben Salem, 2001). Worldwide, approximately 900 000 ha of cactus are cultivated for forage production (Reynolds and Arias, 2001).

In Portugal, several *Opuntia* species have become naturalized (*O. ficus-indica* (L.) Miller, *O. dillenii* (Ker-Gawler) Haw., *O. robusta* H.L. Wendl ex Pfeiffer and *O. elata* Link and Otto ex Salm-Dick), with *O. ficus-indica* the most widespread and economically relevant. This last has two forms, the typical inermis form, *O. ficus-indica* f. *ficus-indica* (L.) Millller, and the rewilded spiny one, *O. ficus-indica* f. *amyclaea* (Ten.) Schelle (Kiesling, 1998) (Fig. 1.1).

In some countries, such as Australia and South Africa, species from the *Opuntia* genus are considered invasive (Julien, 2006; Paterson *et al.*, 2011). In the Iberian Peninsula, *O. dillenii* is the only truly invasive species, even invading areas with very restrictive characteristics (Blasco *et al.*, 2015). On the other hand, *O. ficus-indica* f. *amyclaea* can be considered a species with a weak invasive potential in environments with very specific characteristics. Finally, *O. ficus-indica* has no invasive power, therefore is not dangerous to the Iberian environment or its flora, and it should be considered a naturalized species yet non-invasive (Blasco *et al.*, 2015).

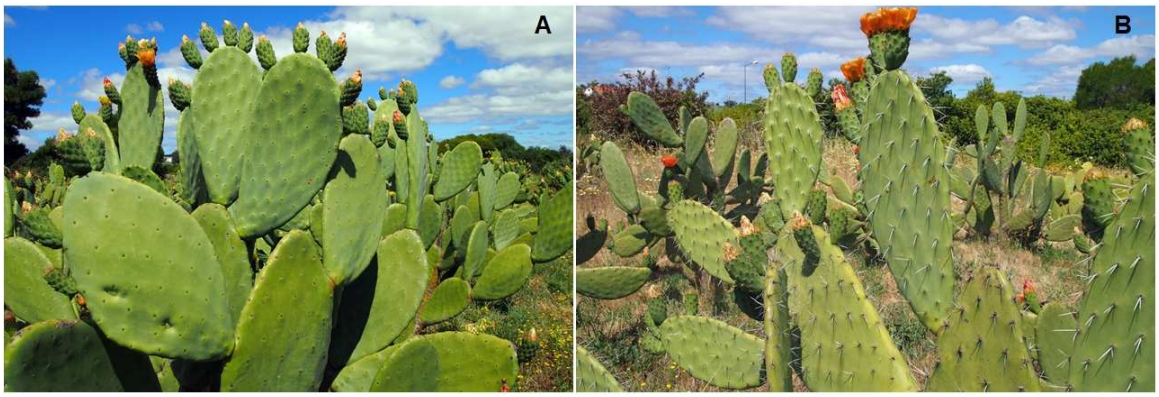

Figure 1.1 *Opuntia ficus-indica*, the inermis form, *O. ficus-indica* f. *ficus-indica* (A), and the rewilded spiny one, *O. ficus-indica* f. *amyclaea* (B).

1.2 Morphology

The morphological features that characterize members of Cactaceae are the presence of short shoots modified into areoles, and in nearly all cacti, inferior ovaries covered by bracts or areoles (Gibson and Nobel, 1986).

The Opuntioideae subfamily differs from all other cacti in having glochids (small, barbed, and deciduous spines) and seeds that are completely enwrapped by a funicular stalk, which becomes hard and bony (Rebman and Pinkava, 2001).

In *Opuntia* spp., the flattened, succulent and articulated shoots are the cladodes (commonly referred to as paddles), and they act as leaves with photosynthetic function. Each cladode in its first year only produces axillary buds called areoles with subtending conic leaves (Fig 1.2A). The areoles produce two kinds of modified into spines leaves: permanent spines with their bases embedded in cork and small, barbed, easily dislodged glochids. The base of the glochid has an abscission layer, easily broken by contact. Areoles can produce long shoots, roots, and flowers (Boke, 1980).

The flowers are large, actinomorphic, sessile, hermaphrodite and solitary and commonly are borne near the apex of the cladodes (Gibson and Nobel 1986). The ovary of the flower is completely embedded within a modified stem termed the receptacle (Fig. 1.2B). The ovary is formed by the fusion of several carpels and consists of a single internal chamber, the locule, where a relatively high number of ovules occur in a parietal location along the ventral wall of the ovary (Boke, 1980) (Fig 1.2B). Cactus flowers have veins from the surrounding stem that enter at the top and sides of the ovary, split and extend upward into the style and downward towards the ovules of the ovary (Rebman and Pinkava, 2001).

Figure 1.2 *Opuntia ficus-indica* small conic deciduous leaves and areolas from a young stem (A), and a detail of a flower where it is possible to observe the receptacle and the high number of ovules (B).

The stamens are indefinite, spirally arranged and have thigmotropic sensitivity (Rosas and Pimienta 1986). Generally, in the far northern hemisphere, anthesis occurs between May and July, while in the southern one, it occurs between February and August.

Autogamic and xenogamic pollination happen in *O. ficus-indica* (Nerd and Mizrahi, 1995). The degree to which the stigmatic area contacts with the anthers influences the probability of selfing. Pollen grains related to self-pollination are found at the stigma base, whereas pollen from other plants is deposited over the stigma (Rosas and Pimienta, 1986).

The *Opuntia* spp. fruits are unilocular and polyspermic (Reyes-Agüero *et al.*, 2006). The fruit is an acrosarcum, a kind of berry derived from an inferior ovary. The fruit is simple and indehiscent, with pericarp undifferentiated (lacking a stony endocarp), surrounded by a fleshy and accrescent exocarp derived from the receptacle (hyphantium) (Stuppy, 2004).

The *Opuntia* spp. have several adaptations that allow them to cope with the lack of available water. Among them are the presence of a shallow, widespread root system and the capacity to quickly produce tiny “rain roots,” which efficiently absorb water after rain but quickly die when water is no longer available. The retention of water within the cactus is enhanced by lowering transpiration rates via 1) having leaves reduced to spines which lowers surface area, 2) having a heavy wax coating (cuticle) on surfaces impeding direct water loss to the atmosphere, 3) having daytime stomata closure, and 4) being succulent (the water adheres to complex carbohydrates called mucilage) (Rebman and Pinkava, 2001).

In *Opuntia* spp., both sexual reproduction and asexual reproduction by seeds and vegetative parts occurs in regions of origin (Pimienta-Barrios and Del Castillo, 2002). However, in the Mediterranean region, the naturalized populations of *Opuntia* facing the difficulties linked to the reproductive process, such as cleistogamy and polyembryony, along with the lack of

rain during summer and decreasing temperatures in autumn, have, essentially, stopped the plants production from seed and limited the extent of new genetic variability (Chessa and Nieddu, 2002).

1.3 Systematics and taxonomy

The Cactaceae family is assigned to the order of Caryophyllales (Wallace and Gibson, 2002) and is one of the most popular, easily recognizable, and morphologically distinct families of plants. The cacti are famous for their beautiful flowers and many bizarre vegetative features but infamous for their formidable nomenclatural and systematic problems (Gibson *et al.*, 1986).

There is no consensus on the number of species and genera to be considered in the Cactaceae family, which is explained by the inadequate knowledge of the existing biodiversity and the difficulties in classifying the highly variable species complexes (Nyffeler and Eggli, 2010). The most recent synoptic family treatments recognize either 1896 species in 127 genera (Anderson, 2001), 1438 species in 124 genera (Hunt, 2006) or 1850 species in 130 genera (Nyffeler and Eggli, 2010).

Traditionally, this diverse family species have been classified into three subfamilies: Cactoideae, Opuntioideae, and Pereskioideae (e.g., Barthlott and Hunt, 1993). Cactoideae encompasses by far the largest share of species and includes the diversity of globular and columnar cacti; Opuntioideae includes the cactus pear (*O. ficus-indica*) and relatives, all characterized by the presence of barbed spines, glochids and seeds enclosed by a bony aril; and, finally, Pereskioideae includes two species-poor genera, *Maihuenia* and *Pereskia* (Nyffeler and Eggli, 2010).

The genus *Maihuenia* has been typically considered a member of Pereskioideae. Recently, its placement in a monogeneric subfamily has been suggested based on its unique ecological and morphological attributes (Anderson, 2001) and molecular phylogenetic analyses (Wallace, 1995a; Wallace, 1995b). However, this new subfamily has not attained consensus, and according to Edwards *et al.* (2005) and Bárcenas *et al.* (2011), a strictly monophyletic classification of the cacti informed by the most recent higher-level analysis would not recognize Maihuenioideae at the same rank as the Cactoideae or Opuntioideae, suggesting that subfamilial rank was assigned prematurely. Phylogeneticists working with cacti, with respect to subfamilial classification and generic delimitation, argue that additional genes and further sampling are necessary before any reclassification of the cacti (Edwards *et al.*, 2005; Griffith and Porter, 2009; Bárcenas *et al.*, 2011).

Nevertheless, in the most recent consensus on generic and species limits for the Cactaceae, Hunt (2006) recognizes four subfamilies, Cactoideae, Opuntioideae, Pereskioideae, and Maihuenioideae. Cactoideae is the largest subfamily, representing seven tribes: Cacteae (25 genera), Cereeae (15), Echinocereeae (25), Hylocereeae (six), Notocacteae (seven), Rhipsalideae (four), and Trichocereeae (23). Opuntioideae is the next largest subfamily with two tribes, Opuntieae and Cylindropuntieae, encompassing ten and seven genera, respectively, and 192 species. The two other subfamilies, Pereskioideae and Maihuenioideae, each consist of a single genus.

Nyffeler and Eggli (2010) reviewed the available molecular-based phylogenetic evidence for characterizing major monophyletic groups and suggested a revised classification of the cactus family into four subfamilies (of which one is paraphyletic), eight tribes (two for Opuntioideae, six for Cactoideae), and six subtribes (for Cactoideae).

The Opuntioideae and Cactoideae subfamilies have long been recognized as monophyletic based on the morphological and molecular data (Barthlott and Hunt, 1993; Nyffeler, 2002; Griffith and Porter, 2009). Opuntioideae members share many structural synapomorphies, such as areoles with glochids, polyporate pollen grains with peculiar exine structures, and seeds surrounded by a funicular cover (Barthlott and Voit, 1979). In addition, studies based on molecular data show that Opuntioideae is characterized by a deletion in the chloroplast genome *accD* region (Wallace, 1995b; Griffith and Porter, 2009).

Tribe Opuntieae consists of seven currently recognized genera, *Brasiliopuntia* A. Berger*, Consolea* Lem*., Miqueliopuntia* Frič ex F. Ritter*, Opuntia* Mill., *Salmiopuntia* Frič, *Tacinga* Britton & Rose, and *Tunilla* D.R. Hunt & Iliff (Majure and Puente, 2014).

The *Opuntia* clade (i.e., *Opuntia* sensu stricto) most likely originated in southern South America during the late Miocene (Arakaki *et al.*, 2011) and from there expanded north into Peru and Ecuador and then into North America, where it became most diverse (Majure and Puente, 2014). Species originating from hybridization and polyploidy account for a large part of the species diversity in *Opuntia* spp. and Opuntieae in general (Majure and Puente, 2014).

Species delimitation within Opuntieae (especially *Opuntia*) is notoriously problematic taxonomically. The number of species in *Opuntia* spp. has been estimated at 250 (Britton and Rose 1919), 160 (Gibson and Nobel, 1986), 181 (Anderson, 2001), and recently 75 (Hunt, 2006). The main reasons for this taxonomical confusion are the diagnostic morphological characters paucity, the high level of phenotypic plasticity, an alleged recent diversification and the prevalent occurrence of hybridization and introgression between sympatric and allopatric species (Wallace and Gibson, 2002; Griffith, 2004; Caruso *et al.*, 2010).

Polyploidy is a common phenomenon throughout tribe Opuntieae, which has been well studied cytologically. In fact, diploids (2*n* = 2*x* = 22) are relatively rare in the tribe, making up only 26.2% of the 164 species with reported chromosome counts. Polyploid taxa within *Opuntia* spp. range from triploid (2*n* = 3*x* = 33) to octoploid (2*n* = 8*x* = 88), and many species have multiple ploidy levels (Pinkaya, 2002; Majure *et al*., 2012).

In Mexico, edible fruits come from wild plants of *Opuntia lindheimeri* Engel., *O. streptacantha* Lem., *O. megacantha* Salm-Dyck, and *O. joconostle* Web. Both *O. amyclaea* Ten., and *O. ficus-indica* are cultivated for fruit production in specialized plantations (Pimienta-Barrios 1990). Natural hybrids are common in both cultivated and wild populations of *Opuntia* spp. (Pimienta-Barrios and Munoz-Urias, 1995). In South America, the United States, Africa, and the Mediterranean Basin, *O. ficus-indica* is the only cultivated species for fruit production. Spontaneous forms have a diploid (2*n* = 2*x* = 22) or tetraploid (2*n* = 4*x* = 44) chromosome number, whereas cultivated varieties have a polyploid (2*n* = 6*x* = 66 or 2*n* = 8*x* = 88) chromosome number (Pimienta-Barrios and Munoz-Urias, 1995). According to various sources cited in the Chromosome Counts Database (CCDB), the number of chromosomes in *O. ficus-indica* varies from the diploid (2*n* = 2*x* = 22) to the octoploid (2*n* = 8*x* = 88), passing through the various intermediate polyploid numbers (Rice *et al.*, 2015).

1.4 Ecophysiology

The Opuntia ecological success and agricultural usefulness in large measure result from the stomatal opening daily pattern. The *Opuntia ficus-indica* plants have nocturnal stomatal opening, with the net CO_2 uptake and water loss occurring during the cooler part of the 24-hour daily cycle. This gas exchange pattern is referred to as Crassulacean acid metabolism (CAM) (Nobel, 1988). The net CO_2 uptake occurs primarily through the stems at night in the Crassulacean acid metabolism (CAM). The CO_2 taken up is incorporated into an organic acid such as malate using the enzyme phosphoenol pyruvate carboxylase (PEPCase), and the accumulating acids are sequestered into the large vacuoles of chlorenchyma cells (Kluge and Ting, 1984). Thus, the chlorenchyma becomes progressively more acidic during the night for CAM plants, and its osmotic pressure also increases (Lüttge and Nobel, 1984). During the day, the acids accumulated the previous night are decarboxylated, and the CO_2 released within the plant is refixed in the stems using 1,5-ribulose bisphosphate carboxylase/oxygenase (Rubisco) via the C3 pathway. The importance of nocturnal stomatal opening and the accompanying net CO_2 uptake regarding the ecological or agronomic success of cacti relates less to the improved CO_2 acquisition than to reduced

water loss. In particular, air and stem temperatures are lower at night, which leads to a lower concentration of water vapour in the stem and hence less water loss for a given degree of stomatal opening; this is important in arid regions (less than 250 mm annual rainfall) and semiarid regions (250 to 450 mm annual rainfall) (Nobel and Bobich, 2002).

In CAM plants, the water use efficiency (the ratio of CO_2 fixed to water loss from transpiration) is about three times higher than it is for highly efficient C4 species such as *Zea mays* and *Saccharum officinarum* and five times higher than for highly efficient C3 species such as *Medicago sativa* and *Triticum aestivum* (Nobel, 1991). Because tissue temperatures in the field tend to average approximately 10°C lower at night than during the daytime, CAM plants tend to lose only 20 to 35% as much water as do C3 or C4 plants for the same degree of stomatal opening during the principal period of net CO_2 uptake (Nobel and Bobich, 2002).

Because of its high drought resistance and high water-use efficiency, cactus pear is frequently cultivated without irrigation. The absolute minimum requirement for rainfed cultivation is *ca.* 200 mm mean annual precipitation, provided that the soil is sandy and deep enough. On silty and loamy soils, the minimum requisite is 300 to 400 mm mean annual precipitation (Inglese, *et al*., 2009). However, in areas with no summer rain, such as the Mediterranean basin, the plants require supplementary irrigation and thinning to get adequate yield and good fruit quality, i.e., 120 g fresh weight (Gugliuzza *et al.* 2002a).

The *Opuntia ficus-indica*, like most cacti, is very sensitive to anoxia in the root zone and therefore cannot withstand any prolonged water logging (Inglese *et al*., 2009), and it is also affected by salinity stress (Gersani *et al.*, 1993).

Extreme temperatures often determine where plant species occur naturally and where they can be cultivated successfully. Freezing temperatures can lead to the formation of extracellular ice crystals, which can disrupt metabolism by cellular dehydration; additionally, intracellular ice crystals can puncture the cell membrane, leading to cell death. High temperatures denature proteins and disrupt membrane integrity (Nobel, 1988). Tolerance of low temperature can be enhanced if the ambient day/night temperature gradually decreases over a period of days, and likewise high temperature tolerance can be increased if the ambient temperature gradually increases; such 'acclimation' or 'hardening' allowing plants to adjust to seasonal changes in air temperature (Levitt, 1980).

Depending somewhat on time of year and particular accessions, mature cladodes can be damaged by air temperatures below -6°C (Goldstein and Nobel, 1994; Nobel, *et al.*, 1995), which severely limits the areas for cultivation of *O. ficus-indica*. On the other hand, mature cladodes of *O. ficus-indica* on properly acclimated plants can survive 60 min at

temperatures exceeding 65°C (Nobel *et al.*, 1986; Nobel, 1988), whereas fruits and roots can be damaged by 60 min below -7°C or above 55°C (Nobel and De La Barrera, 2003). *Opuntia ficus-indica* is absent in regions with less than 350 mm rainfall and daily summer maximum temperature greater than 42°C, with few exceptions (Inglese *et al*. 2009). The optimal day/night temperature regime for nocturnal CO_2 uptake by *O. ficus-indica* is 25/15°C. Higher or lower day/night temperatures result in a sharp decrease of carbon assimilation, leading to poor plant growth and production (Nobel, 1994).

High temperatures are one of the major constraints on the production of high quality fruit in areas with hot and dry summers. High temperature (> 30°C) during the fruit development period shortens the third stage of fruit growth, when most of the growth of edible flesh occurs, leading to advanced and early ripening, with small fruits, low firmness and low sugar content. High temperature during fruit development enhances fruit sensitivity to low (< 8°C) temperature during post-harvest storage, reducing the fruit's post-harvest storage period and shelf-life (Inglese *et al.*, 2002). Still, daily temperatures below 15°C delay fruit ripening time and result in thicker fruit peel and lower soluble solid content and peel colour (Inglese *et al*., 1995; Liguori *et al.*, 2006).

1.5 Germplasm characterization

Knowledge of the existing genetic diversity is extremely important in the conservation, management and efficient utilization of genetic resources. The improvement of crop genetic resources depends on continuous infusions of wild relatives, traditional varieties and the use of modern breeding techniques. These processes all require an assessment of the genetic diversity to select resistant and highly productive varieties (Mondini *et al*., 2009). The assessment of genetic diversity within and between populations could be achieved using morphological, biochemical (allozymes), and molecular-based markers.

Morphological characterization does not imply complex technology; however, considerable physical space is required, and phenotypic plasticity is usually verified by environmental influence. Another disadvantage is that the number of morphological markers is sometimes limited. However, it allows comparison of the agronomic behaviour of different populations subjected to the same environmental pressure (Mondini *et al*., 2009). To standardize and assist in characterization, the International Plant Genetic Resources Institute (IPGRI) has published lists of descriptors for different species, namely, *Opuntia* spp., which are based on the variation of phenotypes such as the characteristics of cladodes, flowers, and fruits (Chessa and Nieddu, 1997).

Allozymes (isozymes) are allelic variants of enzymes encoded by structural genes. Despite the simplicity of their analysis, its main weakness is their relatively low abundance and low level of polymorphism. Moreover, proteins with identical electrophoretic mobility (co-migration) but from distantly related germplasm may not be homologous, and allozymes may be affected by environmental conditions (Kumar *et al*., 2009).

A molecular marker could be defined as a segment of DNA representative of the differences at the genome level. Molecular markers offer numerous advantages over conventional phenotype-based alternatives, as they are stable and detectable in all tissues regardless of growth, differentiation, development, or the defence status of the cell. Additionally, they are not confounded by environmental, pleiotropic or epistatic effects (Agarwal *et al*., 2008).

Molecular markers can be classified into two categories: i) hybridization-based techniques (e.g., restriction fragment length polymorphism, RFLP) and ii) PCR-based techniques (e.g., random amplification of polymorphic DNA, RAPD; amplified fragment length polymorphism, AFLP; inter simple sequence repeats, ISSRs; microsatellites, also known as simple sequence repeats, SSRs, or short tandem repeats, STRs). The molecular techniques differ from each other with respect to important features such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and cost. There are several publications that explain in detail the characteristics, advantages and disadvantages of the different molecular markers (e.g., Agarwal *et al*., 2008; Kumar *et al*., 2009; Mondini *et al*., 2009; Guichoux *et al*., 2011).

Among the different molecular markers, microsatellite sequences are especially suited to distinguishing closely related genotypes because of their high degree of variability. Microsatellites are monotonous repetitions of very short (one to five) nucleotide motifs, which occur as interspersed repetitive elements in all eukaryotic genomes (Tautz and Renz, 1984). Because of their elevated mutation rates, SSRs are typically highly polymorphic, and different individuals exhibit variation manifested as repeat number differences (Guichoux *et al*., 2011).

Microsatellites have been used to evaluate crop germplasm and genetic diversity in several species, namely, rye (Targońska, *et al*., 2016) grape (Muccillo *et al*., 2014), sugarcane (Lu *et al*., 2015), rice (Ahmad *et al*., 2015) and olive (Doveri *et al*., 2008).

Recently, molecular markers have been used in the characterization and evaluation of genetic diversity in populations of *Opuntia* spp. and to clarify some issues of taxonomic nature. In the past two decades, some studies have been conducted to characterize the genetic variability of germplasm collections, identify cultivars, and detect the presence of duplicates using random molecular markers that originate from multiple arbitrary amplicon profiling (Wang *et al*., 1998; Mondragón-Jacobo, 2003; Luna-Paez *et al*., 2007; Zoghlami *et*

al., 2007; García-Zambrano *et al*., 2009; Nagaty and Rifaat, 2012; Valadez-Moctezuma, *et al*., 2015).

Labra *et al.* (2003) documented the usefulness of molecular markers such as AFLP and cpSSR in *Opuntia* species characterization. They found a lack of genetic differentiation between *O. ficus-indica* and *O. megacantha* populations and suggested that *O. ficus-indica* should be considered a domesticated form of the spiny *O. megacantha*.

Griffith (2004) used information from the amplification of the internal transcribed spacer region of nuclear ribosomal DNA (nrITS). They considered the species *O. ficus-indica* to be a group of different clones, selected for their low number of spines and their fleshy fruits that were derived from different parents, most likely from other arborescent opuntias from central and southern Mexico.

Until now, few studies have been conducted to identify genomic microsatellites and to develop markers in *Opuntia* spp*;* Helsen *et al.* (2007) developed 16 SSR markers from *O. echio*s, and Erre *et al*. (2011) obtained ten SSR markers from *O. ficus-indica*. Moreover, two expressed sequence tags were reported by Caruso *et al.* (2010).

Helsen *et al*. (2009) used the previously developed SSR markers (Helsen *et al.*, 2007) to discriminate between two morphologically distinct *O. echios* botanical varieties (*echios* and *gigantea*) native to the Galapagos Islands. However, the current taxonomic differentiation between these taxa was not supported by the molecular data.

Caruso *et al.* (2010) investigated the level of intraspecific genetic diversity among cultivated *O. ficus-indica* varieties and some related species from the Mediterranean region and Mexico by studying 16 SSR (Helsen *et al*., 2007) and 3 EST-SSR polymorphic loci. The clusters identified by their distance and model-based analyses clearly separated the wild opuntias from the cultivated ones. However, the *O. ficus-indica* accessions did not cluster separately from other arborescent cactus pear species such as *O. amyclaea, O. megacantha, O. streptacantha, O. fusicaulis,* and *O. albicarpa*. They also verified that in general, the genotypes cultivated in Mexico showed high levels of diversity, whereas most of the spineless accessions collected in other countries had a very narrow genetic base.

Samah *et al*. (2016) used 13 SSR markers (Helsen *et al*., 2007; Erre *et al*., 2011) to study the genetic diversity of Mexican *Opuntia* germplasm of agronomic and economic importance. The accessions were grouped into five clusters, thus confirming the incorrect delimitation of the species in this genus. The species *Opuntia ficus-indica*, *Opuntia albicarpa*, *Opuntia megacantha*, *Opuntia streptacantha*, *Opuntia lasiacantha*, and *Opuntia hyptiacantha* had no clear boundaries. However, *Opuntia robusta* was separated from the rest of the species. *Opuntia joconostle* and *Opuntia matudae*, which produce acidic fruit, tended to differ from the others.

Most of these reported DNA-based studies have revealed discrepancies between molecular characterization and classical taxonomical classification based on morphological characteristics.

1.6 Germplasm evaluation

Evaluation for biomass production and cladode nutritional value

Opuntia ficus-indica is an important forage crop for livestock feeding in some regions of the world, mainly due to its drought resistance, high biomass yield, high palatability, and adaptability to various soil types (Ben Salem *et al*., 1996).

This species is particularly attractive as feed due to its efficiency at converting water to dry matter (DM) and thus to digestible energy. The most notable *Opuntia* spp. characteristic is its enormous potential to produce large amounts of green succulent fodder, even under relatively unfavourable conditions (Nobel and Bobich, 2002). *Opuntia* spp. can provide a continuous valuable supply of fresh fodder during the dry season given its succulent non-deciduous vegetative structure, a feature rarely found in other forage species (Mondragón-Jacobo and Pérez-González, 2001).

Opuntia ficus-indica dry matter productivity under optimal conditions may reach 45-50 Mg ha^{-1} yr^{-1} (García de Cortázar and Nobel, 1992). Light interception, temperature, soil nutrients, cladode orientation, plant structure, and stem area index are important components that determine cactus productivity (Dubeux *et al.*, 2015). In the semiarid region located in northeast Brazil, in small farms with intensive production systems, it is common to observe dense populations (> 40 000 plants ha^{-1}) of *Opuntia ficus-indica* and *Nopalea cochenillifera* for forage production. In these systems, for well-managed crops DM productivity ranges from 10 to 25 Mg ha^{-1} yr^{-1}, although at research sites productivity up to 30 Mg ha^{-1} yr^{-1} has been registered (Dubeux *et al.*, 2013). In years with severe drought, which often occurs in semiarid regions, maize productivity is close to zero. In this same environment, cactus produces 20-30 Mg ha^{-1} yr^{-1} of dry matter, not to mention 180 Mg ha^{-1} yr^{-1} of stored water in cactus cladodes (Dubeux *et al.*, 2015).

Opuntia species are clonally propagated by placing an unrooted cladode in the soil, which then roots and establishes a new plant. For maximum production in “cut and carry” systems, close spacing of approximately 1.2 x 1.2 m is useful, but where direct grazing is desirable, in-row spacing ranging from 1.0 to 1.5 m and between-row spacing of 3 to 5 m are desirable (Felker *et al.*, 2006). The cladodes of *O. ficus-indica* can be fed to livestock as fresh forage or stored as silage for later feeding.

The nutrient content of *Opuntia* spp. cladodes depends on the genetic characteristics of the species or clone, the cladode's age, the cladode sampling location, the pad harvesting season and the growing conditions, such as soil fertility and climate (Nefzaoui and Ben Salem, 2001; Gugliuzza *et al.*, 2002b; Andrade-Montemayor *et al.*, 2011).

Opuntia ficus-indica offers considerable palatability as well as high mucilage and moisture content, which can serve as a source of potable water for animals (Nefzaoui and Ben Salem, 2001). In normal forage contexts, the high-water content would be a serious disadvantage due to the high cost of transporting such forage. However, during droughts in arid regions, the high moisture content of cactus is an asset because it greatly reduces animal drinking water requirements (Felker *et al.*, 2006).

In general, *Opuntia* cladodes have low levels of dry matter (10–15%), crude protein (4–6%) and neutral detergent fibre (20-31%) (Azócar, 2001; Nefzaoui and Ben Salem, 2001; Costa *et al*., 2012). Besides, it is an excellent energy source, rich in non-fibre carbohydrates (61.7%), and presents a high dry matter digestibility coefficient (Wanderley *et al.*, 2002). The mineral composition is very low in Na, low in P, moderate in Mg, but high in both K and Ca (Retamal *et al.*, 1987; Galizzi *et al.*, 2004).

According Dubeux *et al*. (2015), the nutritive value of *O. ficus-indica* usually is within the range of 40-70 g kg^{-1} for crude protein (CP), 250-300 g kg^{-1} for neutral detergent fibre (NDF), 180-200 g kg^{-1} for acid detergent fibre (ADF), 650-700 g kg^{-1} for total digestible nutrients (TDN), and 500-550 g kg^{-1} for non-fibre carbohydrates (NFC). These values may vary outside of this range depending on the environment and management factors previously mentioned.

Given that the cladodes have low levels of crude protein, fibre, phosphorus, and sodium, they should be combined with other feedstuffs to complete the daily diet (Nefzaoui and Ben Salem, 2001). In the specific case of CP, N fertilization can increase its level up 10%, a level that is normally felt to be necessary for a lactating beef cow (Gonzalez, 1989).

Animals fed exclusively with cactus pear may present weight loss, decreased milk fat and digestive disturbances such as diarrhoea and distended tympanic abdomen (Tegegne, *et al.*, 2007). Cactus pear associated with other fibre sources increases DM levels in the diet and maintains normal conditions in the rumen, thus preventing such undesired effects. According to previous authors, cactus pear could optimally substitute for pasture hay up to 60%, and its inclusion makes a substantial contribution to satisfying the water requirement of sheep.

Cactus pear may substitute for corn meal in the diet of lactating goats without affecting milk production negatively, and it may be an important resource for reducing water intake in dairy goats (Costa *et al.*, 2009).

In synthesis, considering the information obtained on *Opuntia* spp. chemical composition we can conclude that i) cactus pear cannot be fed alone, ii) it must be supplemented with CP and fibre in a mixed diet and iii) it is rich in soluble carbohydrates; thus, adding molasses should be avoided and the amount of grain or other sugar/starch sources in the diet should be limited (Dubeux *et al.*, 2015).

Opuntia ficus-indica plants are pruned annually in plantations for fruit production. Pruning can regulate resource allocation among the various canopy sinks and can maximize light availability within the canopy to support cladode growth, flower bud formation, and fruit growth. Moreover, pruning facilitates pest control, fruit thinning, and fruit harvest (Inglese *et al.*, 2002). This pruned material instead of being discarded as waste can alternatively be used as a valuable feed source for livestock.

As referred before, the nutrient content of *Opuntia* spp. cladodes depends on the genetic characteristics of the species or clone among other factors. Therefore, it seems important to study the nutritive characteristics of the cladodes from Portuguese ecotypes with special emphasis on the spineless plants.

Evaluation for fruit yield and bioactive compounds

Many species in the Cactaceae family produce edible fruit, and among them the genus *Opuntia* spp. has the most relevant role in agriculture. The *Opuntia ficus-indica* produces, in particular, delicious juicy fruits containing a large number of hard seeds, and usually they are eaten raw after being peeled.

Cactus pear is commonly propagated via cuttings, and both single and multiple cladode cuttings are utilized. Single cuttings can be 1 to 2-years-old, and their surface area and dry mass have a significant influence on rooting success and subsequent budding in the field. In Italy, plant spacing ranges from 4 x 6 m (416 plants ha^{-1}) to 5 x 7 m (290 plants ha^{-1}) (Inglese *et al*., 2002). Plants begin to yield 2 to 3 years after planting, reach their maximum potential 6 to 8 years after planting, and bear for 25 to 30 years or even longer depending on pruning and overall orchard management.

Fruit productivity depends on the orchard design, cultural practices, environmental conditions (including soil type) and cultivar (Inglese *et al.*, 2002). Yields of 20 Mg ha^{-1} (Nerd *et al*., 1991), 12-30 Mg ha^{-1} (Barbera *et al.*, 1992), and 17 Mg ha^{-1} (Coetzer and Fouche, 2015) were reported in Israel, Italy and South Africa, respectively.

Average fruit fresh weight and seed weight per fruit vary with cultivar, from 100 to 240 g (Inglese *et al*., 2002) and from 2.0 to 7.0 g (Parish and Felker, 1997), respectively.

The percentage of flesh is less variable than fruit size. It ranges from 60 to 65% for the Italian cultivars “Gialla”, “Bianca”, and “Rossa” (Inglese *et al.*, 1994). Low temperature

during fruit development promotes an increase in peel thickness and a reduction of flesh growth, resulting in a low flesh/peel ratio (Nerd *et al.*, 1991). Fruit quality varies with cultivar and depends on several management, environmental, and physiological factors. Pruning, fruit thinning, and irrigation are the most powerful tools for maximizing fruit size.

Cultivars for fruit production can be differentiated by the colour of the fruit peel and pulp, which can be purple-red, yellow-orange, white-cream or greenish. Red, yellow and white fruits are present in all the cultivated areas, whilst green fruits with white-greenish flesh can be found in Chile and Peru (Mondragón-Jacobo and Pimienta Barrios, 1995). White-flesh fruits are very sensitive to postharvest handling and to specific pests such as the Mediterranean fruit fly (*Ceratitis capitate*) (Inglese *et al*., 2002).

The most appreciated fruits in the international markets, e.g. cv. "Gialla", have yellow-orange flesh, however, consumers unfamiliar with this fruit are highly stimulated by red fruits, which they buy first because of their intense colour (Liguori and Inglese, 2015).

The colour of the fruit is due to the presence of water-soluble nitrogenous pigments (betalains) such as betacyanins (purple-red) and betaxanthines (yellow-orange) (Stintzing *et al.*, 2003), which can be isolated and used as natural food colourants (Saénz, *et al.*, 2009).

The export size fruit must exceed 120 g, the percentage of flesh should not be lower than 55% and harvest sugar values should be at least 13% (Inglese *et al.*, 2002; Felker *et al.*, 2005).

The composition of the fruit varies with ripening. However, as the fruits are non-climacteric, it is important to collect them at the optimal ripeness stage for processing, marketing, or consumption. When the peel colouration is halfway towards that of the fully ripened fruit, the TSS is 12–15 percent depending on the cultivar. At this stage, the fruit is at its best quality for consumption or storage. The sugar, TSS, and vitamin C content increase considerably during the ripening process, while firmness and acidity fall (Sáenz, 2013).

The fruit should be harvested when its peel colour changes, at a time when the umbilical crown is still slightly green. The concentration of reducing sugars should not be less than 13%, and pulp firmness should not be less than 8 kg cm^{-2} (Barbera *et al.*, 1992; Inglese *et al.*, 2002).

Several authors have conducted studies on the chemical composition and antioxidant proprieties of the fruits from *Opuntia* spp., and differences were found both among different *Opuntia* species and among cultivars and ecotypes of *O. ficus-indica* (Kuti, 2004; Medina *et al.*, 2007; Castellanos-Santiago and Yahia, 2008; Chavez-Santoscoy *et al.*, 2009; Guzmán-Maldonado *et al.*, 2010; Cayupán *et al.*, 2011; Dehbi *et al.*, 2013; Nadia *et al.*, 2013; Albano *et al.*, 2015).

In the cactus pear fruit, the acidity is low (0.02%) and the pH high (5.3-6.7) if compared with values found in other common fruit juices. The ascorbic acid content usually varies between 220–260 mg L^{-1} juice, and soluble solids range from 11 to 15°Bx (Stintzing *et al.*, 2003). In fruits of *O. ficus-indica* plants from Argentina, ascorbic acid contents range from 0.26 to 0.48 mg g^{-1} fresh weight. Total phenolic compound content can range between 0.41 and 0.93 mg of gallic acid g^{-1} (Cayupán *et al.*, 2011). Among the transition metals, high manganese content (1.7–2.9 ppm) and good amounts of iron (0.6–1.2 ppm) and zinc (0.3–0.4 ppm) have been found (Gurrieri *et al.*, 2000).

The fruit is a good source of minerals, such as potassium (217 mg 100 g^{-1}), and low in sodium (0.6–1.19 mg 100 g^{-1}), it is also rich in calcium and phosphorus, with levels of 15.4–32.8 mg (100 g^{-1}) and 12.8–27.6 mg (100 g^{-1}), respectively (Sawaya *et al.*, 1983; Sepúlveda and Sáenz, 1990).

Cactus pear (*Opuntia* spp.) fruit has recently gained attention for its nutritional and potential technological values (Piga, 2004). The sweet and juicy cactus pear pulp has interesting health-promoting properties attributed to the presence of certain bioactive compounds. The nutraceutical benefits of *Opuntia* spp. fruits are believed to stem from their alleged antioxidant properties related to ascorbic acid, phenolics including flavonoids, and a mixture of yellow betaxanthin and red betacyanin pigments (Gurrieri *et al.*, 2000; Galati *et al.*, 2003; Tesoriere, *et al.*, 2004; Yahia and Mondragón-Jacobo, 2011; Abdel-Hameed, *et al.*, 2014; El-Mostafa *et al.*, 2014).

1.7 Aims of the study

We live in a changing world in both climatic and economic terms. The Mediterranean region, including mainland Portugal, is a climate change hotspot, and in the latter decades of the 21st century, the Mediterranean is expected to experience the greatest drying among 26 regions across the globe (Giorgi, 2006; Guiot and Cramer, 2016). Global and regional model simulations project a warming scenario with dramatic impacts in the Portuguese region. The near surface temperature increases in Portugal under those scenarios are far higher than the predicted changes in global mean temperature, and translate into dramatic changes of all temperature-related climate indices. Impacts are higher in summer and autumn and in the interior of the country. Concerning precipitation, models project a drier climate, with a shorter and wetter rainy season followed by a long dry summer. The projected reduction in mean precipitation is likely to affect the southern regions of the country more, which already experience a shortage of water and large interannual variability (Miranda *et al.*, 2002).

Given the expected climate changes, there is a growing need to explore the potential of neglected species for future food, fodder, industrial, medicinal, and soil preservation plant resource, suitable for the more affected regions. These neglected species may tolerate stresses such as extreme temperatures, salinity, and drought better than the conventional crops of today. Among these neglected species are members of the Cactaceae family, namely, *Opuntia ficus-indica*, also known as the "prickly pear" or the "cactus pear". *Opuntia* spp. have perfectly adapted to arid zones characterized by droughty conditions, erratic rainfall and poor soils subject to erosion. They thus contribute in periods of drought, serving as lifesaving crops for both humans and animals (Mondragón-Jacobo and Pérez-González, 2001).

In Portugal, *O. ficus-indica* is traditionally cultivated in non-irrigated conditions for edible fresh fruit production and hedge establishment. Recently, some farmers have been focusing on drip-irrigated OFI orchards for fresh fruit production with plant layout and spacing design. These orchards have been carried with both Portuguese ecotypes and improved varieties imported from Italy. However, the Portuguese ecotypes have not yet been characterized, hampering research and breeding efforts directed at the development of improved varieties.

The present study was focused on the following main objectives:

i. To collect and establish Portuguese ecotypes of *Opuntia* spp. in a common garden;
ii. To establish a non-destructive method to estimate the *O. ficus-indica* ecotypes biomass production;
iii. To estimate the plant vigour and biomass production variability among and within ecotypes;
iv. To find populations with biomass production at least like the improved varieties established in the common garden for comparison purposes;
v. To evaluate the nutritional profile of the cladodes from the different Portuguese *O. ficus-indica* spineless ecotypes in comparison with the cultivar "Gialla", and evaluate its potential use as feed for ruminants;
vi. To evaluate the potential of the Portuguese ecotypes for fruit production, in the first years after plantation, in comparison with the Italian cultivars "Bianca" and "Gialla";
vii. To characterize the morphology, the bioactive compounds and the antioxidant properties of *Opuntia* spp. fruits;
viii. To classify different species and ecotypes into distinct groups according to their morphology and fruit chemical characteristics using a multivariate analysis approach;

ix. To improve methods of DNA extraction from *Opuntia* spp. cladodes;

x. To understand the overall pattern of genetic diversity and relationships among germplasm accessions using molecular markers.

1.8 References

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2. Above-ground biomass estimation of *Opuntia ficus-indica* (L.) Mill. for forage crop in a Mediterranean environment by using non-destructive methods

This chapter is based on the following published paper:

Reis, C.M.G., Gazarini, L.C., Fonseca, T.F. & Ribeiro, M.M. (2018). Above-ground biomass estimation of *Opuntia ficus-indica* (L.) Mill. for forage crop in a Mediterranean environment by using non-destructive methods. *Experimental Agriculture*, 54(2), 227-242. https://doi.org/10.1017/S0014479716000211.

2.1 Summary

In marginal lands *Opuntia ficus-indica* could be used as an alternative fruit and forage crop. The plant vigour and the biomass production were evaluated in Portuguese germplasm (15 individuals from 16 ecotypes) by non-destructive methods, 2 years following planting in a marginal soil and dryland conditions. Two Italian cultivars ("Gialla" and "Bianca") were included in the study for comparison purposes. The biomass production and the plant vigour were estimated by measuring the cladodes number and area, and the fresh (FW) and dry weight (DW) per plant. We selected linear models by using the biometric data from 60 cladodes to predict the cladode area, the FW and the DW per plant. Among ecotypes, significant differences were found in the studied biomass-related parameters and several homogeneous groups were established. Four Portuguese ecotypes had higher biomass production than the others, 3.20 Mg ha^{-1} on average, a value not significantly different to the improved "Gialla" cultivar, which averaged 3.87 Mg ha^{-1}. Those ecotypes could be used to start a breeding program and to deploy material for animal feeding and fruit production.

2.2 Introduction

The cactus pear (*Opuntia ficus-indica* (L.) Mill.) (OFI) is a sustainable crop with low input demand and both the cladodes and the fruits can potentially be used as food for humans and animals, particularly in the world's arid and semiarid regions. The OFI ecological and agricultural success is largely due to its Crassulacean acid metabolism (CAM). This photosynthetic pathway is characterized by nocturnal stomata opening with CO_2 uptake primarily occurring from dusk to dawn, while during the day the stomata are closed. This pattern provides higher water use efficiency and annual dry matter productivity than many C3 and C4 cultivated plants (Nobel, 1988).

The Central Mexico is the OFI domestication centre and the species' taxonomic concept may include clones derived from multiple lineages and, therefore, be polyphyletic (Griffith, 2004). The species introduction in the Iberian Peninsula probably occurred at the beginning of the 16th century, after the discovery of America, spreading afterwards throughout the Mediterranean basin (Anderson, 2001).

The Mediterranean region, particularly inland areas, has been suffering from severe drought during extensive summers, and global change is expected to deeply affect this area in the near future. Indeed, global and regional model simulations project a warming scenario with dramatic impacts in this region. The precipitation models anticipate a drier climate with a shorter and wetter rainy season, followed by a long dry summer (Schröter *et al.*, 2005). The cactus pear morpho-physiological characteristics and multiple economic uses represent an alternative crop in this region. The fruit has nutritional and economical value and also represents an interesting crop for small ruminants feeding, by providing energy, water and minerals during periods when food and water are scarce (Andrade-Montemayor *et al.*, 2011; Rodrigues *et al.*, 2016). Additionally, OFI is used to produce natural dyes (Anderson, 2001), as a medicinal plant (Lim, 2012) and the cladodes physicochemical characteristics are suitable for biogas production (Jigar *et al.*, 2011).

In the Mediterranean area, such as Portugal, the OFI is found on roadsides and paths due to its typical ruderal behaviour, and both species forms, *Opuntia ficus-indica* f. *ficus-indica* and *Opuntia ficus-indica* f. *amyclaea* (Ten.) Schelle, can be found. In Portugal, cactus pear is a naturalized species (Inglese *et al.*, 2009) and it is cultivated for edible fruit production and hedges establishment. The local ecotypes have variability in the plant vigour, the shape of the cladodes, the presence or absence of spines, the spine length, the corolla colour, the pulp colour and the fruit ripening time (unpublished results).

The large stems (cladodes) are the main photosynthetic organs in OFI. The light interception, the CO_2 uptake and, ultimately, the OFI productivity depend on the stem area

index (both sides of the cladode surface per ground area), which is the equivalent to the leaf area index (Nobel, 1988). The genotypes with faster photosynthetic area growth have higher light interception capacity and, therefore, higher potential and earlier capacity for fruit production (Caloggero and Parera, 2004). Some authors computed the photosynthetic area by establishing a linear relationship between the cladode area and the maximum length and width (Caloggero and Parera, 2004; Sáiz and Fernandez, 1990; Tiznado-Hernandez *et al.*, 2010). The cladode fresh weight (FW) was estimated using the linear relationship between the FW and the product of the cladode maximum length by its width and mean thickness (Pinto *et al.*, 2002).

To the best of our knowledge, few studies to estimate the biomass production by nondestructive methods have been reported to date on this species for the Mediterranean region. To estimate the dry weight (DW) two studies obtained linear regression models that relate the cladode DW with the product of the cladode maximum length by its width and diameter of the neck (Curt *et al.*, 2011; Sáiz and Fernández, 1990) for this crop in Spain. The morphology and potential biomass production of the Portuguese *Opuntia* germplasm is unknown. Using 16 OFI Portuguese ecotypes established in a common garden situated in inland Portugal, the objectives of the study were: (1) to establish a non-destructive method to estimate biomass production, (2) to estimate the plant vigour and the biomass production variability among and within populationsn and (3) to find populations with biomass production at least similar to the improved varieties established in the common garden for comparison purposes.

2.3 Materials and Methods

Plant material and experimental design

A mission to collect Portuguese OFI germplasm took place in the early spring of 2012. The cladodes were sampled from 15 individuals in 16 different ecotypes/populations, encompassing various altitudinal levels (Table 2.1). They were located in the Central and Southern regions of inland Portugal, and one in the Madeira Island (Table 2.1). Two improved Italian cultivars (cv.), “Bianca” and “Gialla”, were included for comparison purposes, OFI-06B and OFI-07G respectively. The mature cladodes were single-planted during May 2012 at the School of Agriculture in Castelo Branco, Portugal (39º49’17”N; 7º27’41”W, elevation 365 m). The plant spacing was 1.5 × 2.5 m (2667 plants ha^{-1}). The experimental design was a randomized complete block design for the 18 populations, with three replicates and five plants each replicate – the elementary plot, 2.5 × 1.5 × 5 = 18.75 m^2. In the experiment borders rows were planted to eliminate side effects.

Table 2.1 Identification and origin of the studied *O. ficus-indica* populations.

Population	Origin	Altitude (m)	Geographic coordinates	
			Latitude	Longitude
OFI-01	Alcochete	25	38°43'32.14"N	8°57'58.22"W
OFI-03	Cascais, Guincho	185	38°45'23.18"N	9°27'38.48"W
OFI-04	Portalegre	372	39°16'22.45"N	7°26'13.12"W
OFI-05	Arronches	293	39° 5'21.06"N	7°12'7.05"W
OFI-06 (B)	Italy	--	--	--
OFI-07 (G)	Italy	--	--	--
OFI-08	Melides	29	38° 8'28.91"N	8°44'14.28"W
OFI-09	Santo André	25	38° 4'38.13"N	8°46'38.08"W
OFI-11	Albufeira	61	37° 5'23.33"N	8°17'27.03"W
OFI-12	Cacela-a-Velha	20	37° 9'22.50"N	7°32'47.98"W
OFI-13	Monforte da Beira	260	39°45'8.34"N	7°16'54.83"W
OFI-14	Idanha-a-Velha	275	39°59'57.30"N	7° 9'3.51"W
OFI-15	Ponte de Sor	125	39°16'15.45"N	8° 0'44.72"W
OFI-16	Biscainho, Coruche	76	38°54'40.93"N	8°37'17.00"W
OFI-17	Castelo Branco	402	39°48'58.84"N	7°29'37.85"W
OFI-18	Reguengos Monsaraz	223	38°27'27.04"N	7°39'21.77"W
OFI-19	Concavada, Alvega	105	39°27'15.96"N	8° 3'51.88"W
OFI-20	Madeira	116	32°38'54.18"N	16°57'46.38"W

B – cv. “Bianca”; G – cv. “Gialla”.

The provenance trial was planted in a granitic soil, with pH 5.9 and low organic matter content; a marginal soil with reduced overall soil profile depth and low water holding capacity. Fertilizers with nitrogen, phosphorus and potassium were applied, 40 kg ha^{-1} each element, to reduce possible differences in soil fertility, but no irrigation or tillage was used. The weeds were controlled by mechanical mowing. The Köppen–Geiger climate classification for Castelo Branco is Csa. The average annual temperature was 15.4 °C (Fig. 2.1) during the period of the experiment (2012–2014). The driest and hottest months were July and August with average temperatures above 24 ºC and absolute values reaching 41 ºC. The coldest months were December, January and February, with average temperatures below 10 ºC. The mean number of days with temperature equal or below 0 °C was 19, ranging between 0 °C and −5.2 ºC. In winter the rainfall was much higher than in summer – typical for Mediterranean climate – with the highest precipitation in October and November (Fig. 2.1). The vegetative growth of OFI occurred during the time of the year when the precipitation was lower.

Model construction and evaluation

In March 2014, 60 cladodes (ca. three per population) aged from 1- to 2-years-old, were sampled in plants from all the 18 populations established in the experimental field. Besides the cladodes scanned images, the area (*A*, cm^2), the FW (g) and the DW (g) were recorded.

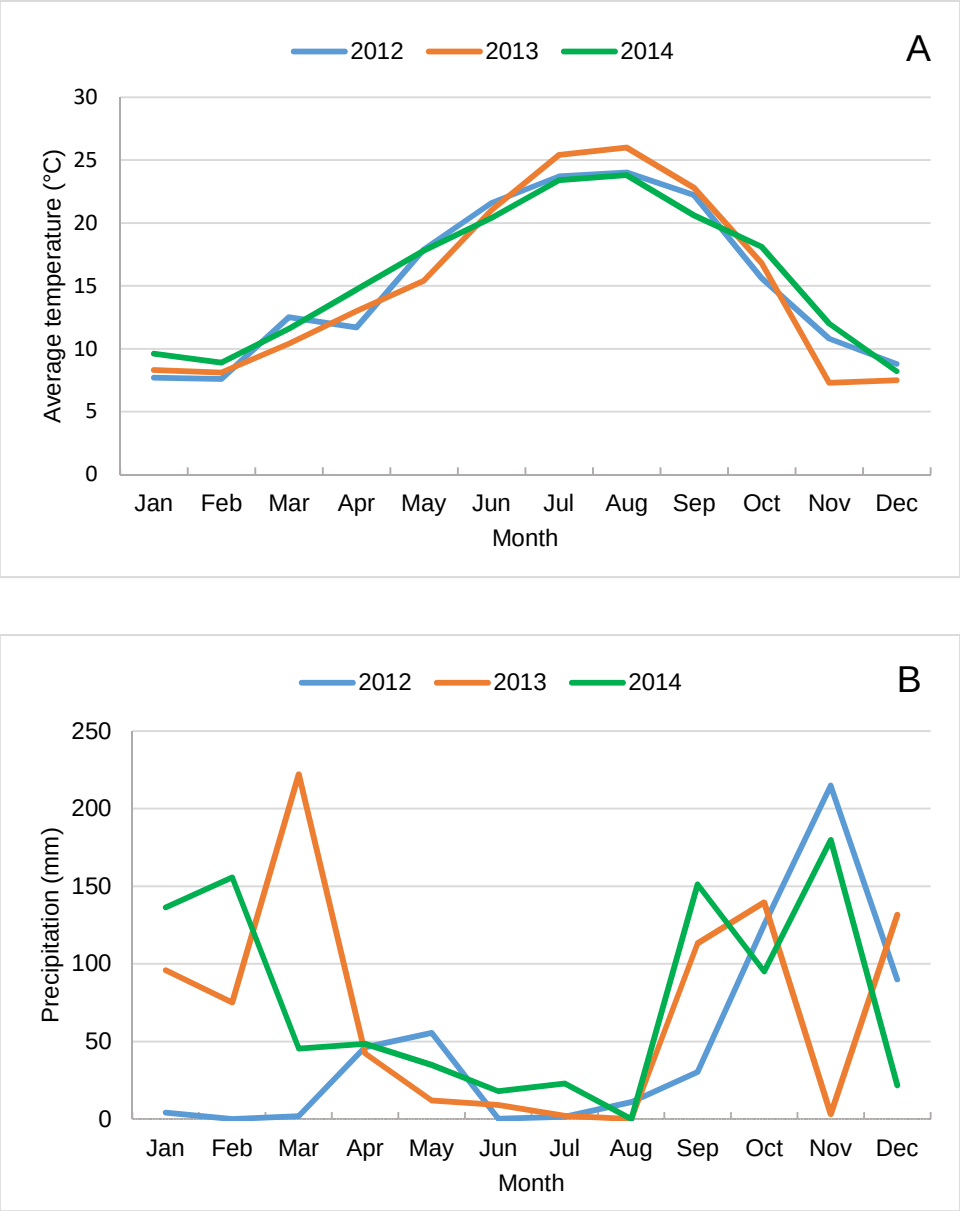

Figure 2.1 Average temperatures (A) and accumulated precipitation (B), in the region of Castelo Branco, for the 3-years period, 2012-2014.

The following biometric parameters were measured in each cladode: the length (*L*, cm), the width (*W*, cm), the mean thickness (*T*, cm) and the diameter of the neck (*D*, cm). The length and the width were measured using a graduated ruler. The average thickness per cladode was computed using three measurements of the cladode thickness, at the apex and on both sides at the maximum width point, using a digital caliper (T1, T2 and T3 in Fig. 2.2). The neck diameter (*D*) was also determined with a digital caliper (Fig. 2.2). The cladode area (*CA*, cm^2) was quantified using two methods, the image analysis based on the software Image J v.1.49b (Rasband, U. S. National Institutes of Health, Bethesda, Maryland, USA), and the cladode weight paper silhouettes, according to Garcia de Cortázar and Nobel

(1992). The FW was measured with a precision scale. Afterwards, each one of the 60 cladodes was fragmented and dried (65 ºC for 72 h) to obtain the respective DW.

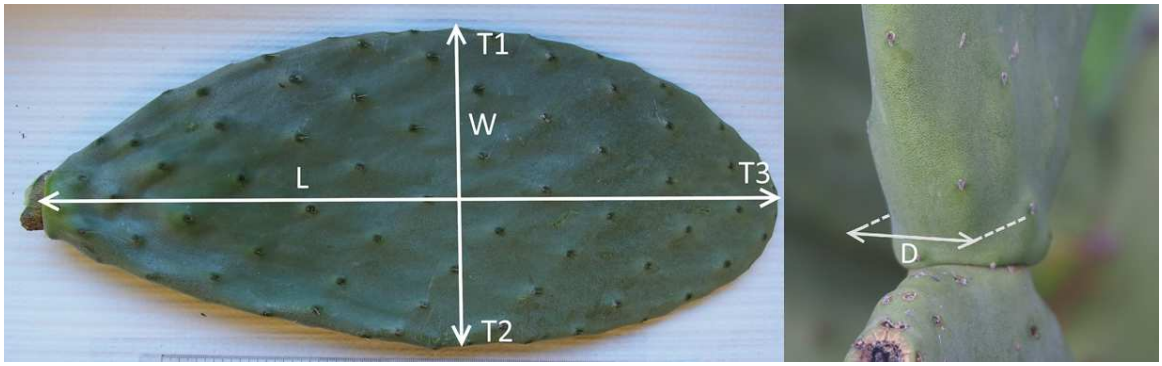

Figure 2.2 Biometric parameters measured in the *O. ficus*-indica cladodes. *L* - Length; *W* - Width; *T* - Mean thickness (obtained from the arithmetic mean of points T1, T2 and T3); *D* - Diameter of the neck.

The relationship among the cladode area (*CA*, cm^2), the FW (g), the DW (g) and the potential regressor variables, '*X*' as *D*, *L*, *T* and *W*, were analysed both graphically and statistically in order to develop predictive models for the biomass production estimation by means of non-destructive methods. We tested 5, 18 and 7 candidate models, for each of the response variable '*Y*', *CA*, *FW* and *DW*, respectively (detailed in Table S2.1, Appendix 1). The linear regression model assumptions were verified, including linear relationship between the variables *Y* and *X*, homoscedasticity (equal error variances), normality and independence of errors. The first three assumptions were both graphically and statistically validated. The Durbin–Watson test for autocorrelation was selected to validate the fourth assumption, the errors independence, by assuming a first-order autoregressive error model, which is consistent to the time series dataset. The model selection and validation was based on the fitting and the prediction ability of those candidate models by using the coefficient of determination (R^2) and the root mean square error (*RMSE*) to assess the criteria quality and the estimates precision, respectively (Montgomery *et al.*, 2012).

Germplasm evaluation

In April 2013 (330 days after plantation, DAP) and March 2014 (660 DAP) the cladode number per plant (*CNp*) was recorded in the 15 individuals from each one of the 18 populations (16 ecotypes plus the cultivars "Gialla" and "Bianca"). The length, the maximum width, the mean thickness and the neck diameter were measured in all the cladodes of the 15 individuals per population, according the procedures outlined in the previous section.

For the biomass production assessment, the Cladodes Area (*CAp*, m^2), the *FWp* (kg) and the *DWp* (kg), were estimated using regression models.

The selected linear models were used to estimate the studied ecotypes' biomass. After fitting the models, the Shapiro–Wilk test for normality and the Levene's test of equality of variances were applied. The data were subsequently analysed using the one-way ANOVA, followed by pairwise comparisons using either the Tukey or the Games-Howell (in the absence of homoscedasticity) post hoc tests. With an absence of normality of the distributions, the non-parametric Kruskal–Wallis test was used to compare the means, followed by pairwise comparisons using Dunn's procedure with a Bonferroni correction for multiple comparisons. The statistical significance was accepted with a probability of type I error of 5%, for both the omnibus test and the multiple comparisons. The statistical analyses were performed using the IBM SPSS Statistics software v.21 (IBM Corp., NY.).

2.4 Results

Characterization of the supporting database and model selection

The summary statistics of the 60 cladodes used to develop the linear models and to estimate the cladode area and the FW are shown in Table 2.2 Since no significant differences were found between the two methods used to estimate the area of the cladodes, the image analysis and the weight of the cladodes paper silhouette, t (118 df) = 0.076, p = 0.94, we have chosen the former, because it is easier and faster to use than the paper silhouette method.

Table 2.2 Summary characteristics recorded in the *O. ficus-indica* cladodes used to develop the linear models (n = 60 obs.).

Statistic	Length (*L*, cm)	Width (*W*, cm)	Mean thickness (*T*, cm)	Cladode area (*CA*, cm^2)	Fresh weight (*FW*, g)	Dry weight (*DW*, g)	Dry matter (%)
Mean	38.61	18.70	1.86	586.71	893.93	66.28	7.49
SD	4.69	2.48	0.28	86.70	173.01	11.80	0.77
Min	30.00	13.00	1.42	440.68	583.14	45.44	5.75
Max	48.00	24.50	2.53	845.75	1267.60	99.55	10.60
CV (%)	12.14	13.25	15.13	14.78	19.35	17.80	10.28

SD – standard deviation; CV (%) – coefficient of variation; Min – minimum; Max – maximum.

The three models for each dependent variable (*CA*, *FW* and *DW*, in Table 2.3) were selected from the candidate models (described in Table S2.1, Appendix 1) based on the evaluation statistics for the quality of the fit and the predictive performance detailed in the Material and Methods section.

Table 2.3 List of the selected equations to predict the *CA*, *FW* and *DW* in *O. ficus-indica*. Coefficients, standard errors and root mean square errors (RMSE) of linear regression models.

Equation	Mathematical model	n	Model*	a	b	R^2	*RMSE*
(1)	$CA = 48.13+0.75\ (L \times W)$	60	2	48.13 (22.92)	0.75 (0.03)	0.91	26.68
(2)	$FW = 36.91+0.64\ (L \times W \times T)$	60	10	36.91 (35.97)	0.64 (0.03)	0.91	52.26
(3)	$DW = 8.49+0.49\ (W \times T \times D)$	58	28	8.49 (4.79)	0.49 (0.04)	0.72	6.05

CA - area of the cladode, one face (cm^2); *FW* – cladode fresh weight (g); *DW* – cladode dry weight (g); *D* – diameter of the neck (cm); *L* - cladode length (cm); *T* – cladode average thickness (cm) and *W* – cladode maximum width (cm). * Model numbering in the S2.1 table.

The graphical and the statistical analyses of the response variables and the available potential regressors (interactions between the *X*'s variables and transformations of the original variables) indicated that the interaction variables $L \times W$ and $L \times W \times T$ were good predictors of the cladode area (Fig. 2.3A) and the FW (Fig. 2.3B), respectively. The assumptions of linearity, independence of errors, homoscedasticity, outliers and normality of the residuals were met. The linear regression established that the product $L \times W$ could predict the cladode area (*CA*, one face, cm^2), F (1 df, 58 df) = 565.03, $p < 0.05$, and the product $L \times W \times T$ could predict the cladode FW (g), F (1 df, 58 df) = 588.52, $p < 0.05$. The highest R^2 was 0.91 for both the *CA* and *FW* estimation models (Table 2.3, equations 1 and 2, respectively), meaning that 91% of *CA* and *FW* observed variation was explained by the models. Also, both models displayed the lowest *RMSE* values (26.68 and 52.26, respectively) compared to the other assayed models' values (Table S2.1, Appendix 1).
In the case of the DW, the product $W \times T \times D$ could predict the cladode DW (g), F (1 df, 56 df) = 146.12, $p < 0.05$ (Fig. 2.3C). Considering the *DW* estimation model, the highest coefficient of determination was 0.72, the *RMSE* value 6.05 and 72.3% of the observed variation was explained by equation 3 (Table 2.3).

Biomass production assessment

The number of cladodes per plant was recorded 330 and 660 DAP (year 1 and year 2, respectively), and the non-destructive biomass quantification of 18 populations was estimated using the selected equations (Table 2.3). The population with the highest number of cladodes per plant was cv. "Gialla" with 5.1 and 25.9 cladodes in year 1 and 2, respectively, and the ecotype OFI-18 had the lowest value, i.e. 1.9 and 6.2 cladodes in year 1 and 2 respectively (Fig. 2.4A).

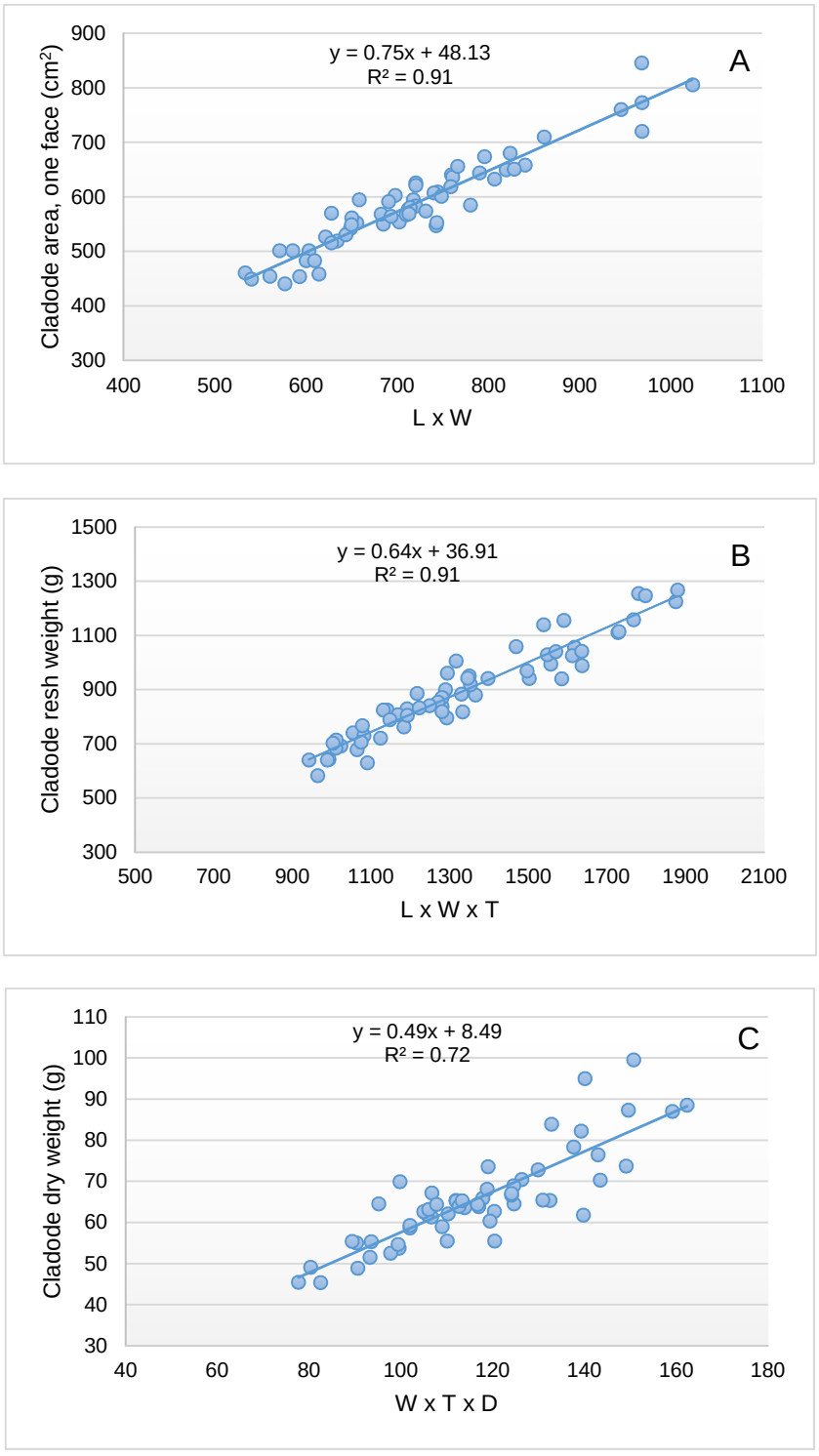

Figure 2.3 Scatter plots and lines of the three chosen allometric equations to estimate: (A) the cladode area *CA* = 48.13 + 0.75 (*L* × *W*), (B) the fresh weight *FW* = 36.91 + 0.64 (*L* × *W* × *T*), and (C) the dry weight *DW* = 8.49 + 0.49 (*W* × *T* × *D*). The length (*L*), width (*W*) thickness (*T*) of the cladode and diameter of the neck (*D*) were the predictor variables used.

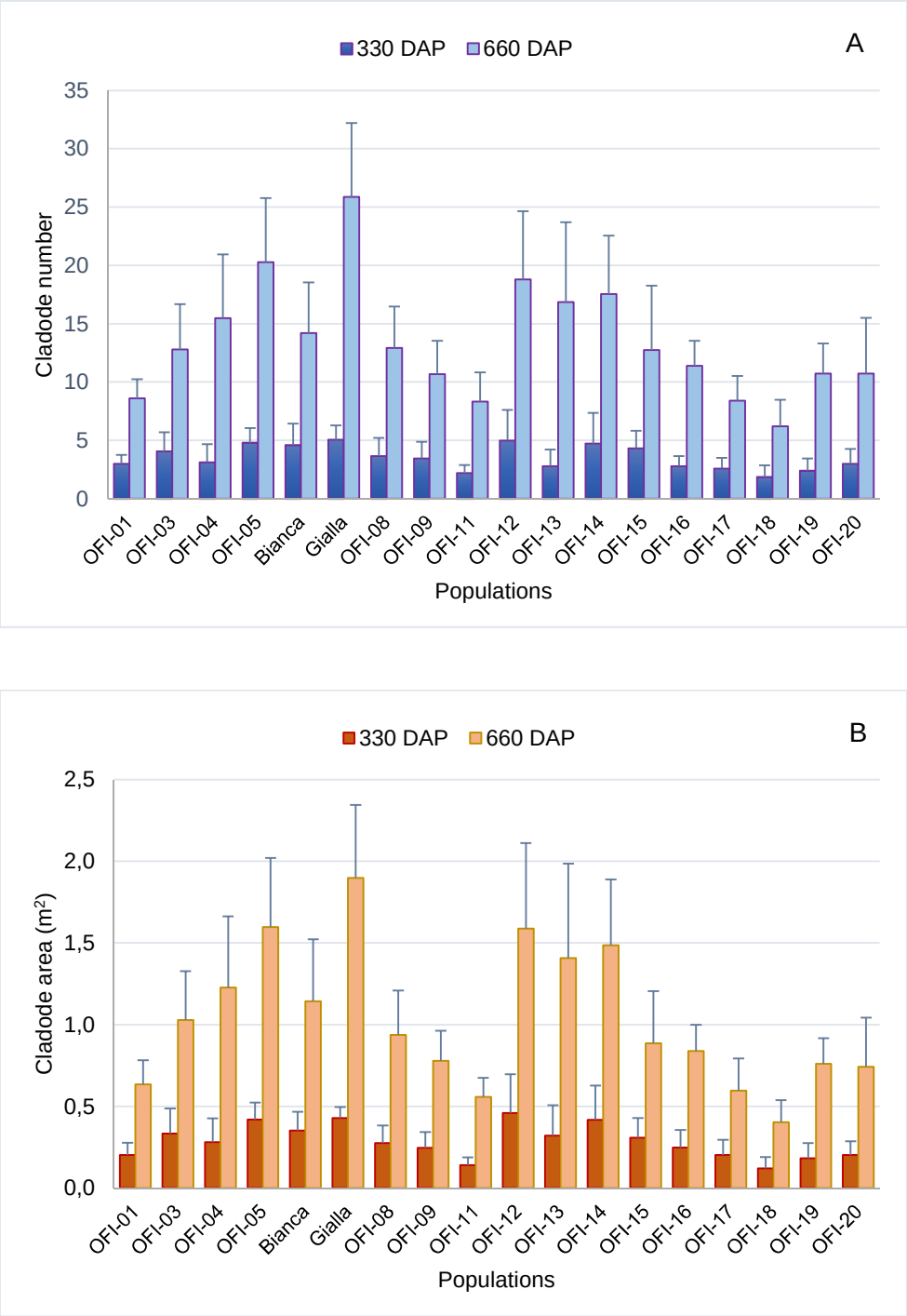

Figure 2.4 Average number of cladodes per plant (*CNp*) (A) and average cladode area per plant (*CAp*) (B) in the 18 populations of *O. ficus-indica* studied, 330 and 660 days after plantation (n=15 plants per population). The *CAp* values were estimated from the allometric equation: $CA = 48.13 + 0.75 (L \times W)$.

Considering all the 18 populations, the mean was 3.5 cladodes per plant in year 1 and this value increased more than 3.5-fold in year 2 (mean 13.5 cladodes per plant). The number of cladodes per plant was not normality distributed (all populations) according to the

Shapiro–Wilk test, hence the nonparametric Kruskal–Wallis test was used to test for differences among populations. This test showed significant differences in the number of cladodes per plant among the 18 OFI populations in year 1 (χ^2 (17) = 89.85, $p < 0.05$, n = 270) and in year 2 (χ^2 (17) = 148.09, $p < 0.05$, n = 270). The pairwise comparisons revealed five and four homogeneous groups in year 1 and 2, respectively. Thus, statistically significant differences in the number of cladodes among some of the populations can be inferred, e.g. OFI-7 from OFI-18 (Fig. S2.1, Appendix 2).

Considering the CA per plant, both faces, the highest values were 0.46 m^2 in the ecotype OFI-12 (year 1) and 1.90 m^2 in the cv. "Gialla" (year 2). The lowest value was observed in the ecotype OFI-18, with 0.12m^2 and 0.40m^2 in year 1 and 2 respectively (Fig. 2.4B). The mean value for the area of cladodes per plant increased ca. 3.5-fold from year 1 (0.29 m^2) to year 2 (1.03 m^2).

The "Gialla" cultivar also displayed the highest FW per plant in both years (3.94 kg and 14.16 kg, year 1 and 2, respectively) and the lowest values were found again in the OFI-18 ecotype (1.16 to 4.19 kg, year 1 and 2, respectively) (Fig. 2.5A). The mean *FWp* values for all the populations increased ca. 3.5-fold from year 1 (2.56 kg) to year 2 (8.84 kg).

The highest DW values per plant were found in OFI-12 (0.33 kg) in year 1 and in cv. "Gialla" (1.45 kg) in year 2. The lowest DW values per plant were observed in ecotype OFI-18 (0.11 kg and 0.43 kg, year 1 and year 2, respectively) (Fig. 2.5B). The mean *DWp* values also increased around 4-fold from year 1 (0.22 kg) to year 2 (0.86 kg).

The normality tests, the standardized skewness and the Shapiro–Wilk test, for variables cladode area, FW and DW per plant indicated that the data were normally distributed. The Levene's F test revealed lack of homogeneity of variances ($p < 0.05$), thus the Welch's F test was used. The one-way ANOVA revealed the existence of significant differences among the 18 populations for the three variables (*CAp*, *FWp* and *DWp*) (Table 2.4). The Games–Howell post hoc test showed significant differences among the OFI populations, and the group including the cv. "Gialla" plus the OFI-05, OFI-12, OFI-13 and OFI-14 ecotypes produced higher photosynthetic area than the remaining populations (Table S2.2, Appendix 3). In the case of both the fresh and DW per plant, the Games–Howell post hoc tests revealed significant differences among the OFI populations and it could be concluded that the group constituted by the two improved cultivars. ("Gialla" and "Bianca"), and the ecotypes OFI-04, OFI-05, OFI-12, OFI-13 and OFI-14 outperformed the remaining populations concerning the fresh and DW per plant (Tables S2.3 and S2.4, Appendices 4 and 5, respectively). From year 1 to 2, the number of cladodes per plant, the photosynthetic area, the FW and the DW per plant increased, on average, in a ratio superior to 3.5.

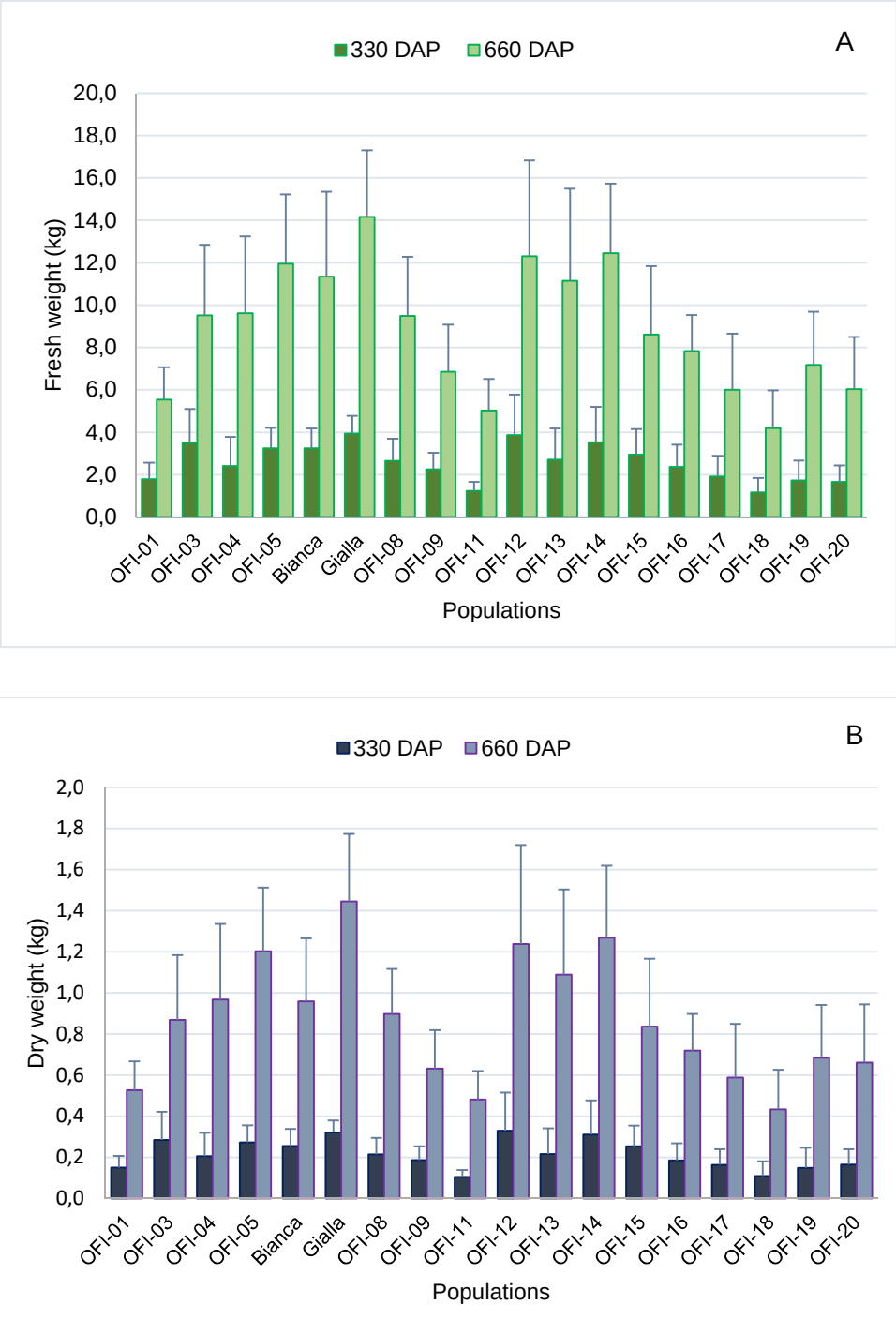

Figure 2.5 Average fresh weight per plant (*FWp*) (A) and average dry weight per plant (*DWp*) (B) in the 18 populations of *O. ficus-indica* studied, 330 and 660 days after plantation (n=15 plants per population). *FWp* and *DWp* were estimated from the allometric equations: $FW = 36.91 + 0.64 (L \times W \times T)$ and $DW = 8.49 + 0.49 (W \times T \times D)$, respectively.

Table 2.4 Welch's ANOVA statistic for the area of cladodes (*CAp*, m^2), fresh weight (*FWp*, kg) and dry weight (*DWp*, kg) per plant. Sig.=significance.

Biomass parameters	Year	Welch's statistic	df_1	df_2	Sig.
Area of cladodes per plant (*CAp*)	Year 1	17.73	17	93.66	$p < 0.05$
	Year 2	25.69	17	93.60	$p < 0.05$
Fresh weigh per plant (*FWp*)	Year 1	14.36	17	93.61	$p < 0.05$
	Year 2	16.79	17	93.71	$p < 0.05$
Dry weight per plant (*DWp*)	Year 1	13.55	17	93.52	$p < 0.05$
	Year 2	16.88	17	93.69	$p < 0.05$

Table 2.5 ANOVA results (α = 0.05) for the 10 populations of *O. ficus-indica* with the higher biomass production 660 days after plantation (year 2) (n=15 plants per population).

Biomass Parameter	Sum of squares	Degrees of freedom (df)	Mean square	F	Sig.
Number of cladodes per plant (*CNp*)	2371.76	9	263.53	9.65	0.00
Area of cladodes per plant (*CAp*)	14.68	9	1.63	9.32	0.00
Fresh weight per plant (*FWp*)	403.18	9	44.80	3.46	0.00
Dry weight per plant (*DWp*)	5.61	9	0.62	5.13	0.00

A one-way ANOVA was made for the 10 populations with higher biomass production in year 2 (OFI-03, OFI-04, OFI-05, OFI-08, OFI-12, OFI-13, OFI-14, OFI-15, cv. "Bianca" and cv. "Gialla"), and significant differences were found for the variables *CNp*, *CAp*, *FWp*, *DWp* (Table 2.5). The Tukey's multiple comparisons of means produced six, eight, three and five homogeneous groups for the number of cladodes, area of cladodes, FW and DW per plant respectively (Table 2.6). The ecotype OFI-05 did not differ from the cv. "Gialla" with respect to the number of cladodes per plant. The ecotypes OFI-05, OFI-12 OFI-13 and OFI-14 were not significantly different from the cv. "Gialla" for the variables *CAp*, *FWp* and *DWp*. Those four ecotypes have few spines, the cladode shape is ovate and the petal colour is yellow, similar to the cv. "Gialla".

2.5 Discussion

Prediction models

We developed linear models to estimate the cladode area, the FW and the DW in Portuguese OFI ecotypes established in a common garden under similar climate conditions, using non-destructive measurements. The variables (*L*, *W*, *T* and *D*) used in the models were also used in similar studies, but in other populations grown in different climatic

conditions (Caloggero and Parera, 2004; Curt *et al.*, 2011; Pinto *et al.*, 2002; Sáiz and Fernández, 1990). In the linear models reported herein, the variables $L \times W$ and $L \times W \times T$ were good predictors of the cladode area and the FW, respectively. The regression and the correlation coefficients' values obtained for the area of the cladode (respectively 0.75 and 0.91) were similar to those reported by Caloggero and Parera (2004) in OFI populations in Argentina [$CA = 6.31 + 0.8\ (L \times W)$, $R^2 = 0.93$]. However, this comparative analysis should be done with caution due to the lack of some information namely the coefficients' standard errors. A comparative analysis with other models previously published to predict the fresh and DW is not possible (Curt *et al.*, 2011; Pinto *et al.*, 2002; Sáiz and Fernández, 1990) as they lack the intercept (regression through the origin) and the R^2 value does not have the same statistical significance (Montgomery *et al.*, 2012).

The linear model suggested by Sáiz and Fernández (1990) and Curt *et al.,* 2011) for the *DW* estimation, using the empirical relationship between *DW* and the regressor, $W \times L \times D$, gave no satisfactory results with our data, explaining only 52.7% of the DW variability. In the case of the *DW* estimation, the interaction term $W \times T \times D$ was considered the best regressor term amongst the set of potential variables, explaining 72.3% of the DW variability.

Table 2.6. Mean values and standard deviation for cladode number, area of cladodes, fresh weight and dry weight per plant in the group with 10 populations of *O. ficus-indica* with higher biomass production, in the 660 days after plantation (year 2) (n=15 plants per population).

Population	*CNp*	*CAp* (m^2)	*FWp* (kg)	*DWp* (kg)	Spines	Cladode shape	Petal color
OFI-03	12.8 (3.88)^{cd}	1.03 (0.30)^{cde}	9.52 (3.33)^{b}	0.87 (0.32)^{bc}	Interm	Ellip	Scarl
OFI-04	15.5 (5.49)^{bcd}	1.23 (0.43)^{bcde}	9.61 (3.63)^{b}	0.97 (0.37)^{bc}	Few	Ovate	Yell
OFI-05	20.3 (5.51)^{ab}	1.60 (0.42)^{ab}	11.95 (3.27)^{ab}	1.20 (0.31)^{abc}	Few	Ovate	Yell
OFI-06B	14.2 (4.33)^{bcd}	1.14 (0.38)^{bcde}	11.34 (4.01)^{ab}	0.96 (0.31)^{bc}	Interm	Ellip	Scarl
OFI-07G	25.9 (6.33)^{a}	1.90 (0.45)^{a}	14.16 (3.15)^{a}	1.45 (0.33)^{a}	Few	Ovate	Yell
OFI-08	12.9 (3.53)^{cd}	0.94 (0.27)^{de}	9.50 (2.78)^{b}	0.90 (0.22)^{bc}	Interm	Ellip	Scarl
OFI-12	18.8 (5.86)^{bc}	1.59 (0.52)^{ab}	12.30 (4.53)^{ab}	1.24 (0.48)^{abc}	Few	Ovate	Yell
OFI-13	16.9 (6.83)^{bcd}	1.41 (0.58)^{abcd}	11.14 (4.35)^{ab}	1.09 (0.42)^{abc}	Few	Ovate	Yell
OFI-14	17.5 (5.01)^{bcd}	1.49 (0.40)^{abc}	12.45 (3.29)^{ab}	1.27 (0.35)^{ab}	Few	Ovate	Yell
OFI-15	12.3 (4.50)^{d}	0.89 (0.32)^{e}	8.61 (3.22)^{b}	0.84 (0.33)^{c}	Interm	Ellip	Scarl

CNp – cladode number per plant; *CAp* – area of cladodes per plant (m^2); *DW* – dry weight per plant (kg); *FWp* – fresh weight per plant (kg); B – cv. "Bianca"; G – cv. "Gialla"; Interm – intermediate; Ellip – elliptic; Scarl –scarlet; Yell - yellow. Means followed by the same letter do not differ significantly (ANOVA and Tukey post hoc test, $p = 0.05$).

Biomass production assessment

Considering the number of cladodes per plant, the photosynthetic area, the FW and the DW per plant, the cv. “Gialla” outperformed the other populations, which reflects its origin as improved material. Caloggero and Parera (2004) also reported the superior performance of this cultivar compared to the Argentinean ecotypes, considering as parameters the number of cladodes per plant and photosynthetic area.

The number of cladodes per plant observed in cv. “Gialla” at 330 and 660 DAP (5.1 and 25.9, respectively) are similar to those presented by Calogero and Parera (2004), who reported values 6.2 and 23.2 at 330 and 450 DAP, respectively. In the case of the area of cladodes per plant, the observed values in the cv. “Gialla” at 330 and 660 DAP (0.43 and 1.90, respectively) are also similar to those reported by Calogero and Parera (2004), i.e. 0.4 and 1.7 at 330 and 450 DAP, respectively.

Without a model to predict the dry matter, the alternative is to use a destructive sample of cladodes, a time-consuming process. Besides, the dry matter needs to be evaluated several times, for this parameter varies during the year. Neder *et al.* (2013), using the path analysis, demonstrated that a correlation exists between fresh and dry matter production, thus an indirect selection for this trait based on the fresh matter production and the photosynthetic area is possible. Nevertheless, we have developed a linear model to estimate the cladode DW explaining 72.3% of its variability, using the interaction term between width and mean thickness of the cladode times the diameter of the neck ($W \times T \times D$).

Considering the group of the 16 OFI Portuguese ecotypes, a significant genetic variability in plant growth rate was found, as revealed by biomass production. Four ecotypes (OFI-05, OFI-012, OFI-13 and OFI-14) out of the 16 Portuguese ecotypes evaluated for biomass production were not significantly different from the cv. “Gialla”. Furthermore, these results indicate that a clonal selection program should be started using clones to produce fodder and fruit. Additionally, the selected four ecotypes were identified as *O. ficus-indica* f. *ficus-indica* and had few spines like the cv. “Gialla”.

The studied ecotypes were collected in locations with different altitude, but no relationship between the biomass production and the geographic origin of the ecotypes was found. The only pattern detected is related to the cladode shape, since the ecotypes with ovate cladodes produced higher biomass compared to those with elliptical cladodes.

A dry matter productivity of 3.9 Mg ha^{-1} for a density of 2667 plants ha^{-1} (0.27 plants m^{-2}) in the 2nd year after planting was obtained in the cv. “Gialla”. The mean value for the dry matter productivity was 3.2 Mg ha^{-1} for the top Portuguese ecotypes group (OFI-05, OFI-012, OFI-13 and OFI-14). Garcia de Cortázar and Nobel (1992) obtained a productivity of 5.5 Mg DW ha^{-1} $year^{-1}$ during the 2nd year after planting, under irrigation and density of

0.25 plants m^{-2}. The observed differences are explained by the dryland conditions verified in our study, since the water availability plays a primary role in the OFI carbon gain. Herein, the vegetative growth of OFI occurred during the time of the year with low rainfall and in a soil with low water holding capacity. The cactus pear is extremely tolerant to high air temperatures, but cladodes can be damaged by air temperatures below −6°C, depending on particular genotypes (Goldstein and Nobel, 1994; Nobel and De la Barrera, 2003; Valdez-Cepeda *et al.*, 2001). Such sensitivity to low temperatures severely limits the areas for OFI cultivation in temperate regions. Nevertheless, the probability of temperatures below −6 ºC is close to zero in Castelo Branco region, according to meteorological records from the last 30 years (AEMET, 2011), with the mean temperature values being within the OFI cultivation requests.

Assuming a density of 5000 plants ha^{-1} in dryland farming conditions, we expect a FW biomass production of 60–70 Mg ha^{-1} (nearly 6–7 Mg ha^{-1} of dry matter for a dry matter content of about 10%) in the 2nd year after plantation.

In the present study, due to logistic conditions, we used a single cladode per hole. In fact, the biomass production is affected by the number of cladodes per hole. In the establishment of an orchard it is advisable to plant two (or more) cladodes per hole, for this will increase the number of cladodes per plant and the photosynthetic area and, afterwards, the earlier fruiting potential after planting (Caloggero and Parera, 2004; Inglese *et al.*, 2002).

The lack of variance homogeneity in the four of the studied variables, *NCp*, *CAp*, *FWp* and *DWp*, was registered among some of the 18 OFI populations, which reflects the existence of inter-populations variability. The provenance test was installed using cuttings and the intra-population variability detected in the first two years after planting may be related to phenotypic variations in single cuttings before planting. Indeed, single cuttings can be one- to two-years-old, and their surface area, dry mass and environmental factors, such as soil fertility and water availability, have a significant influence on successful rooting and subsequent performance in the field (Inglese *et al.*, 2002). The hypothesis that the observed variability is the result of somaclonal mutation does not seem very likely, as shown by Zoghlami *et al.* (2012), who observed the genetic stability of long-term micropropagated OFI plantlets. However, we should not to exclude that in some ecotypes, the intra-population variability might be due to the existence of polyclonality. As stated by Griffith (2004), the actual taxonomic concept of OFI may include clones derived from multiple lineages and be polyphyletic. The origin of the observed intra-population variability should be clarified through the use of molecular markers.

Climate change is expected to deeply affect the Mediterranean region, particularly inland areas in the near future. The OFI, by its morpho-physiological characteristics and multiple

economic uses, represent an alternative crop in the Mediterranean region. We have developed linear models to estimate the cladodes photosynthetic area, the fresh and dry matter production by a non-destructive method. Significant variability in biomass production among the studied populations of OFI was found in this study. Some ecotypes showed low vegetative vigour and have reduced interest as material for vegetative propagation. Thus, the proper choice of cultivars or ecotypes for clonal propagation in new plantations either for animal feed or fruit production is a key factor. Within the 16 evaluated Portuguese OFI ecotypes it was possible to select four ecotypes with similar biomass production to the “Gialla” cultivar. They constitute an interesting plant material to initiate a breeding program through clonal selection, either for fodder and/or fruit production. Further studies are ongoing to evaluate the flowering, fruiting and nutritional characterization of the Portuguese ecotypes, using qualitative and quantitative approaches.

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3. Fruit production from Portuguese ecotypes of *Opuntia ficus-indica* in comparison to commercial Italian clones

This chapter is based on the following paper in press:

Reis, C.M.G., Gazarini, L.C. & Ribeiro, M.M. Fruit production from *Opuntia ficus-indica* ecotypes in comparison to commercial Italian clones. *Horticultural Science.* In press.

3.1 Summary

Fruit production, as an elementary chemical characteristic of the fruit, was evaluated in 16 *O. ficus-indica* Portuguese ecotypes cultivated in a marginal soil without tillage, in the second and third years after plantation. The *Opuntia ficus-indica* ecotypes were compared with the Italian cultivars “Bianca” and “Gialla”. Significant differences were found among the *O. ficus-indica* ecotypes in biomass-related parameters and fruit yield, and different groups were established. Two spineless ecotypes (OFI-12 and OFI-13) had highest biomass production, with 9.9 Mg ha^{-1} dry matter on average. This was not significantly different from the “Gialla” cultivar, which averaged 11.9 Mg ha^{-1}, for a density of 2667 plants ha^{-1}, in the third year after plantation. Among Portuguese ecotypes, the fruit yields ranged from 2.4 to 10.1 Mg ha^{-1} fresh weight. The cultivars “Gialla” and “Bianca” had the highest fruit yield (13.8 and 13.6 Mg ha^{-1} fresh weight, respectively). The “Gialla” cultivar and the group of ecotypes with orange pulp produced fruits had a larger size and weight compared to the “Bianca” cultivar and the group of ecotypes with white pulp.

3.2 Introduction

The cactus pear, *Opuntia ficus-indica* (L.) Miller (OFI), is a long-domesticated cactus crop that is important in agricultural economies throughout arid and semiarid parts of the world. It is a species of the Cactaceae family, and was originally domesticated in Mexico (Griffith 2004). The OFI has particular morphological and physiological characteristics that allow high water use efficiency. The specialized photosynthetic system in cacti, the Crassulacean acid metabolism (CAM), enables greater water to dry matter conversion than C3 and C4 photosynthetic pathways (Nobel 1988; Han and Felker 1997).

Prickly-pear cacti were brought to Europe by the first Spanish conquerors between the end of the 15th century and the beginning of the 16th century (Barbera *et al.,* 1992). This species is an alternative to that cultured in the inland areas of the Mediterranean peninsula, where climate change is predicted to have a higher impact (Schröter *et al.*, 2005).

Italy (mainly Sicily), with 7400 ha and 78 000 tons is the main cactus pear fruit producer in Europe (Albano *et al.*, 2015). At least 4000 ha of specialized plantations produce 60 000 tons of fruits (Inglese *et al.*, 2010), which corresponds to an average production of 15 tons per hectare. In Portugal, OFI is traditionally cultivated in non-irrigated conditions for edible fresh fruit production and the establishment of hedges, but recently some farmers have begun focusing on drip irrigated OFI orchards for fresh fruit production, with a specific plant layout and spacing design. In 2016, the area occupied by specialized plantations was approximately 820 ha, with a likely increase in the near future. The OFI local ecotypes display variability in plant vigour and biomass production (Reis *et al.*, 2018), and some producers have been using them in orchard plantations for fruit production.

According to Inglese *et al.* (2002), OFI plants begin to yield 2 to 3 years after planting, reaching their maximum potential 6 to 8 years after planting, and bearing for 25 to 30 years or even longer, depending on pruning and overall orchard management.

The fruits of *Opuntia* spp. have nutraceutical benefits that are believed to stem from their antioxidant properties, which are related to ascorbic acid; phenolic compounds, including flavonoids and betalains; and a mixture of yellow betaxanthin and red betacyanin pigments (Galati *et al.*, 2003; Stintzing *et al.*, 2005).

Plant genetic resources play a significant role in the improvement of cultivated plants; however, germplasm utility depends on its evaluation. To our knowledge, there is no data available regarding the fruit production potential from the Portuguese OFI ecotypes. The main objectives of this study were to: (i) evaluate 16 Portuguese OFI ecotypes for their fruit production and the elemental characteristics of the fruit in the second and third years after plantation, in a marginal soil with no-tillage farming, (ii) evaluate the same OFI ecotypes for

their biomass production in the third year after plantation and (iii) compare the results with two improved Italian cultivars, “Bianca” and “Gialla”.

3.3 Material and methods

Plant material and experimental design

The OFI plants were planted in a provenance trial at the School of Agriculture of Castelo Branco, Portugal (39º49' N; 7º27' W, elev. 365 m a.s.l.) in May 2012. Sixteen Portuguese ecotypes of *O. ficus-indica* and two improved Italian cultivars “Bianca” and “Gialla”, which were included for comparison purposes, were studied (Table 3.1). The plant spacing was 1.5 × 2.5 m (2667 plants ha^{-1}). The experimental design was a randomized complete block design, with three replicates and five plants in each replicate (2.5 × 1.5 × 5 = 18.75 m^2). The provenance trial was planted in a marginal soil, with a reduced overall soil profile depth and low water holding capacity. The soil was granitic, with pH 5.9 and a low organic matter content. Nitrogen (N), phosphorus (P) and potassium (K) (40 kg ha^{-1} each) fertilizers were applied annually to reduce possible differences in soil fertility. Irrigation was applied during the summer period (approximately 60 mm). No tillage was used, and weeds were controlled by mechanical mowing. Cladode pruning and flower thinning were conducted, resulting in no more than six fruits on each fruit-bearing cladode.

Table 3.1 Identification, origin and morphological description of the *Opuntia ficus-indica* (OFI) populations.

Population	Origin	Altitude (m)	Cladode shape	Spines	Petal colour	Fruit	
						Shape	Pulp colour
OFI-01	Alcochete	25	Elliptic	Many	Scarlet	Elliptic	White
OFI-03	Cascais	185	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-04	Portalegre	372	Ovate	Few	Yellow	Ovoid	Pale Yellow
OFI-05	Arronches	293	Ovate	Few	Yellow	Ovoid	Orange
OFI-08	Melides	29	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-09	Santo André	25	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-11	Albufeira	61	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-12	Cacela-a-Velha	20	Ovate	Few	Yellow	Ovoid	Orange
OFI-13	Monforte da Beira	260	Ovate	Few	Yellow	Ovoid	Orange
OFI-14	Idanha-a-Velha	275	Ovate	Few	Yellow	Ovoid	Orange
OFI-15	Ponte de Sor	125	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-16	Coruche	76	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-17	Castelo Branco	402	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-18	Reg. Monsaraz	223	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-19	Alvega	105	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI-20	Madeira	116	Ovate	Few	Yellow	Ovoid	Orange
OFI “Bianca”	Italy	--	Elliptic	Intermediate	Scarlet	Elliptic	White
OFI “Gialla”	Italy	--	Ovate	Few	Yellow	Ovoid	Orange

The Köppen-Geiger climate classification of Castelo Branco is Csa. The average annual temperature in 2014 and 2015 was 15.8 and 16.2 °C, respectively. The driest and hottest months were July and August, with mean temperatures close to 24 ºC and absolute values reaching 40 ºC. The coldest months were January, February and December, each of which had average temperatures below 10 ºC. The mean number of days with a temperature equal or below to 0°C was 30, with a range between 0 and -5.4 ºC. The winter rainfall was much higher than in summer, which is typical for a Mediterranean climate, with the highest precipitation in October, November and December. The accumulated precipitation in 2014 and 2015 was 433 and 910 mm, respectively. These values were significantly different from the average over the last 30-years of 735 mm. The *Opuntia. ficus-indica* vegetative growth occurred during the time of the year when the precipitation was lower.

Determination of pH, acidity, total soluble solids and dry matter of the fruit

Three replicates, including ten fruits of each ecotype, were sampled. The peel was manually removed and the pulp was briefly homogenized in a kitchen-type blender. Afterward, the pulp was separated from the seeds, portioned, and stored at −80 °C until analysis. After defrosting, the juice was centrifuged at 14 000 rpm for 10 min and the supernatant was used for the determination of pH, acidity, and total soluble solids (TSS, %). Total acidity was determined using a pH meter (after the titration of 10 ml of seedless pulp-juice against 0.01 N sodium hydroxide (NaOH) to the end point (pH 8.2), and the results were expressed as percentage citric acid. The total dry matter (DM, %) was determined in three replicates of five fruits according to AOAC (2000). Triplicate readings were taken for each sample.

Morphological characterization

A basic morphological characterization of the plants was made using the following qualitative descriptors: cladode shape, number of spines, petal colour, fruit shape and pulp colour (Chessa and Nieddu 1997).

Evaluation of fruit and biomass production

All of the fruits from the 15 plants in each population were collected and weighed at full maturity by the end of August and beginning of September in 2014 and 2015 (780 and 1 140 days after plantation, DAP), respectively. The fruit production per plant (*FPp*, kg), the number of fruits per plant (*FNp*) and the distribution of fruits among two weight classes (*FWc*) were evaluated for each of the 18 populations (Fig. 3.1).

In April 2015 (1 050 DAP) the cladode number per plant (*CNp*) was recorded in the 15 individuals from each of the 18 populations. The length (*L,* cm), maximum width (*W*, cm),

mean thickness (*T*, cm), and the diameter of the neck (*D*, cm), were measured in all cladodes of the 15 individuals per population. The biomass production assessment was made by a determination of the cladode area (*CAp*, m^2), fresh weight (*FWp*, kg) and dry weight per plant (*DWp,* kg) using the following regression models (Reis *et al.*, 2018):

1) $CAp = 48.13 + 0.75\ (L \times W)$, ($R^2 = 0.91$)
2) $FWp = 36.91 + 0.64\ (L \times W \times T)$, ($R^2 = 0.91$)
3) $DWp = 8.49 + 0.49\ (W \times T \times D)$, ($R^2 = 0.72$)

Figure 3.1 General view of the provenance trial in the third year after plantation (A) and a detail of *O. ficus-indica* fruits at commercial maturity (B).

Statistical analysis

The data was analysed using a one-way analysis of variance (ANOVA) followed by pairwise comparisons using the Tukey or the Games-Howell (in the absence of homoscedasticity) post hoc tests. The statistical significance was accepted, with a probability of a type I error of 5%, for both the omnibus test and the multiple comparisons. The statistical analyses were performed using SPSS Statistics software v.21 (IBM Corp., Armonk, NY, USA).

3.4 Results and discussion

Morphological characterization

With regard to the morphological characteristics of the ecotypes, the cladode shape was elliptic or ovate, the number of spines could be categorized into one of three classes (many, intermediate or few), the flowers had two types of petal colour (scarlet or yellow), the fruit was elliptic or ovoid and the pulp colour was white, pale yellow or orange (Table 3.1). The ecotype OFI-01 corresponds to the *Opuntia ficus-indica* f. *amyclaea* (Ten.) Schelle due to

the high number of spines, while all the remaining ecotypes belong to the *O. ficus-indica* f. *ficus-indica* (L.) Miller according to previously published criteria (Kiesling, 1995).

The ecotype OFI-04 differs from the others in the pulp colour, and in other morphological and chemical characteristics of the fruit. Assuming the criteria that an *Opuntia* variety could be defined as one that was indistinguishable from other strains based on the internal or external appearance of the fruit or overall plant morphology (Felker *et al*., 2005), the ecotype OFI-04 could be considered a new variety.

Elementary chemical characteristic of the fruit

The OFI populations were significantly different with regard to the DM content of the fruits, F (17, 34.24) = 31.61, $p < 0.05$, which varied between 13.8% (OFI-20) and 17.9% (OFI-16) (Table 3.2). The DM of the fruits of the "Bianca" and "Gialla" cultivars were 15.6 and 16.1%, respectively. The average DM of the fruit in the OFI ecotypes of the provenance trial was 15.7%. The TSS content of the cactus pear varied from 13.05% (OFI-20) to 15.63% (OFI-03) (Table 3.2) and significant differences were found among ecotypes, F (17, 36) = 29.20, $p < 0.05$. The pH varied from 6.03 (OFI-13) to 6.47 (OFI-16), and the acidity values ranged between 0.05 and 0.07% citric acid (Table 3.2).

Table 3.2 Fruit dry matter (DM %), acidity (% citric acid), pH and total soluble solids (TSS %) from the juice of the different cactus pear populations. Values are means ± standard deviation (n = 30, each sample was analysed in triplicate).

Population	Fruit DM (%)	Juice		
		pH	Acidity (% citric acid)	TSS (%)
OFI-01	15.87 ± 1.02	6.30 ± 0.00	0.05 ± 0.00	14.25 ± 0.28
OFI-03	16.31 ± 0.21	6.30 ± 0.00	0.05 ± 0.00	15.63 ± 0.15
OFI-04	16.53 ± 0.39	6.10 ± 0.00	0.05 ± 0.00	15.10 ± 0.10
OFI-05	16.17 ± 0.24	6.10 ± 0.00	0.06 ± 0.00	15.12 ± 0.28
OFI-08	15.92 ± 0.34	6.20 ± 0.00	0.07 ± 0.00	13.70 ± 0.15
OFI-09	14.96 ± 0.41	6.27 ± 0.06	0.06 ± 0.00	14.10 ± 0.41
OFI-11	14.72 ± 0.55	6.20 ± 0.00	0.06 ± 0.00	13.55 ± 0.35
OFI-12	16.46 ± 0.38	6.17 ± 0.06	0.05 ± 0.00	15.07 ± 0.31
OFI-13	15.98 ± 0.09	6.03 ± 0.03	0.06 ± 0.00	15.05 ± 0.28
OFI-14	16.16 ± 0.74	6.20 ± 0.00	0.06 ± 0.00	14.65 ± 0.09
OFI-15	15.25 ± 0.37	6.27 ± 0.06	0.05 ± 0.00	13.47 ± 0.08
OFI-16	17.90 ± 0.85	6.47 ± 0.06	0.05 ± 0.00	15.10 ± 0.26
OFI-17	14.77 ± 0.55	6.33 ± 0.06	0.05 ± 0.00	14.35 ± 0.30
OFI-18	15.00 ± 0.15	6.20 ± 0.00	0.06 ± 0.00	13.23 ± 0.12
OFI-19	15.41 ± 0.40	6.30 ± 0.00	0.06 ± 0.00	14.37 ± 0.34
OFI-20	13.81 ± 0.29	6.17 ± 0.06	0.06 ± 0.00	13.05 ± 0.13
OFI, "Bianca"	15.63 ± 0.34	6.40 ± 0.00	0.07 ± 0.00	13.72 ± 0.20
OFI, "Gialla"	16.06 ± 0.44	6.10 ± 0.00	0.06 ± 0.00	14.67 ± 0.19

The acidity values were determined by titration and the results are equivalent to citric acid. Among all the OFI populations, the average pH and acidity values were 6.23 and 0.06% citric acid, respectively. We can assume that the observed variations in the TSS and the other variables we studied reflected differences at the genotype level, because the ecotypes were grown in a similar soil and climate conditions and the fruits were harvested when in the same physiological state. The maturity indices for cactus pear include TSS values between 13 and 17%, pH between 6.0 and 6.5, and titratable acidity between 0.03 and 0.12% (Inglese and Gugliuzza 2002; Inglese *et al.*, 2002). All the ecotypes investigated in the current study had TSS values higher than 13%. The pH and acidity values were in agreement with those reported in previous studies (Albano *et al*., 2015; Medina *et al.,* 2007).

Biomass production

The largest cladode area, *CAp*, was 4.34 m^2 in both OFI-13 and the "Gialla" cultivar, and the lowest value of 1.27 m^2 was observed in the ecotype OFI-18 (Fig. 3.2).

The "Gialla" cultivar had the highest fresh weight per plant, *FWp*, (40.79 kg) followed by the ecotypes OFI-13 and OFI-12 (37.32 and 37.19 kg, respectively), and the lowest value was found in the ecotype OFI-18 (11.21 kg) (Fig. 3.2). The "Gialla" cultivar had the highest dry weight per plant, *DWp* (4.48 kg), followed by the ecotypes OFI-13 and OFI-12 (3.84 and 3.62 kg, respectively), and the lowest value was observed again in ecotype OFI-18 (1.12 kg) (Fig. 3.2).

The one-way ANOVA revealed significant differences among the 18 ecotypes for the three variables (*CAp*, *FWp* and *DWp*) (Table 3.3). The Games–Howell post hoc test revealed significant differences among the OFI populations, with the group including the "Gialla" cultivar and the OFI-04, OFI-05, OFI-12, OFI-13 and OFI-14 ecotypes having a higher photosynthetic area compared to the other populations. For both the *FWp* and *DWp*, the Games–Howell post hoc tests revealed significant differences among the OFI populations, with the group constituted by the "Gialla" cultivar and the ecotypes OFI-12, OFI-13 and OFI-14 outperforming the other populations.

The dry weight had a significant correlation with the *CAp* (r^2 = 0.984) and *FWp* (r^2 = 0.993), while the *FPp* had a significant correlation with the *FNp* (r^2 = 0.941).

A DM productivity of 11.95 Mg ha^{-1}, for a density of 2 667 plants ha^{-1} (0.27 plants m^{-2}), in the third year after planting, was obtained in the "Gialla" cultivar, based on the biomass of the cladodes. The ecotypes OFI-13 and OFI-12 did not differ significantly from the "Gialla" cultivar and a DM productivity of 10.24 and 9.65 Mg ha^{-1} was observed under the same conditions, respectively. Due to their spineless nature, they could be used to start a breeding program to deploy material for animal feeding or young cladode production.

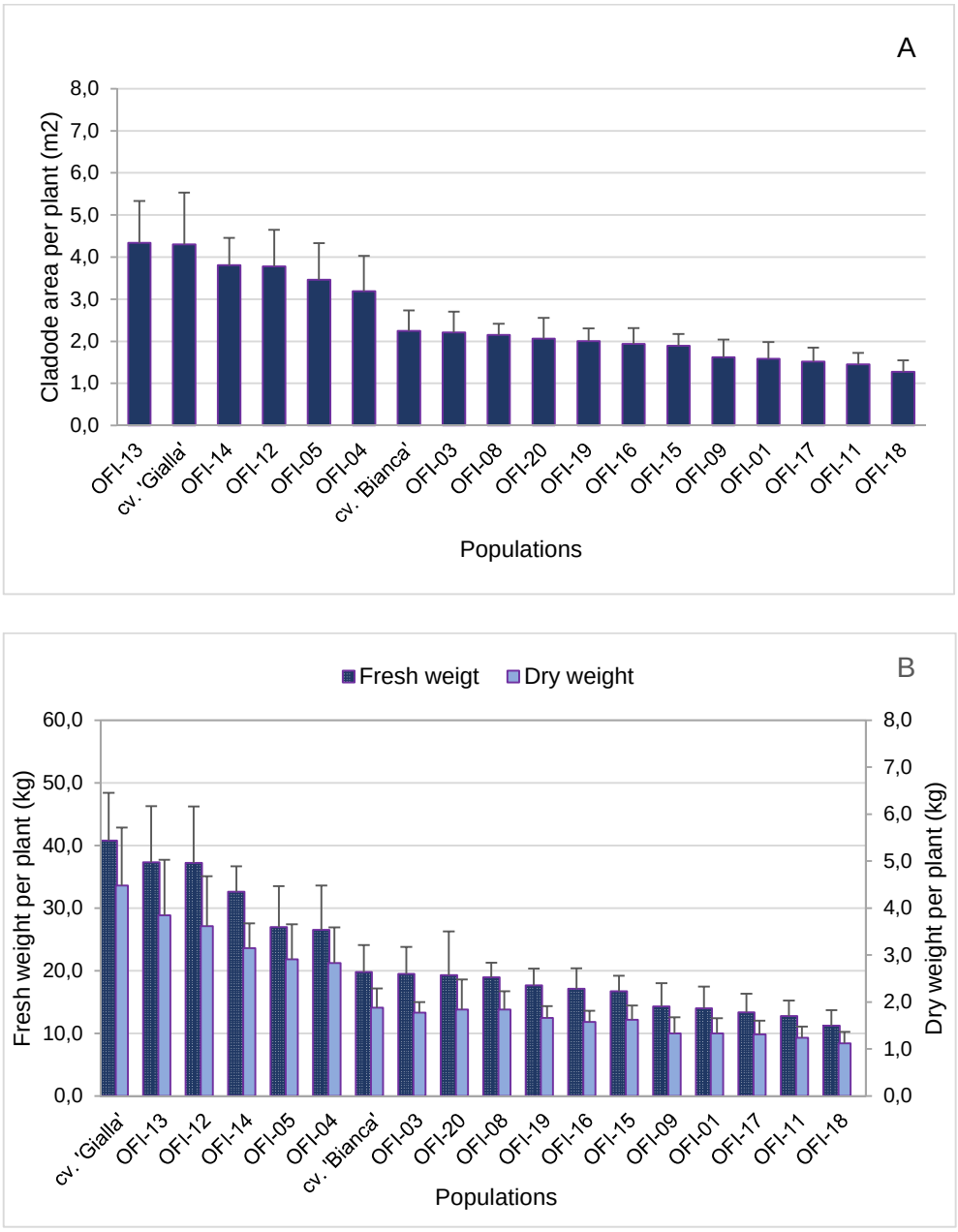

Figure 3.2 Cladode area per plant (A) and fresh and dry weight per plant (B) in the 18 populations of *Opuntia ficus-indica* (OFI) studied, 1050 days after planting (year 3) (*n* = 15 plants per population).

Table 3.3 Welch's analysis of variance (ANOVA) statistic for the area of cladodes (CAp, m^2), fresh weight (FWp, kg) dry weight (DWp, kg), fruit number (FNp) and fruit production (FPp) per plant), in the third year after planting.

Biomass and fruit production parameters	Welch's statistic	df_1	df_2	Sig.
Area of cladodes per plant (*CAp*)	31.98	17	92.85	$p < 0.05$
Fresh weigh per plant (*FWp*)	40.63	17	92.84	$p < 0.05$
Dry weight per plant (*DWp*)	29.23	17	92.82	$p < 0.05$
Fruit number per plant (*FNp*)	35.26	17	13.29	$p < 0.05$
Fruit production per plant (*FPp*)	28.54	17	13.38	$p < 0.05$

Fruit production

The number of fruits and fruit production per plant (kg) in the second and third years after plantation are shown in Fig. 3.3. There was a marked difference in the number of fruits and in the production of fruit per plant when comparing years 2 and 3. In year 2, the fruit production was almost irrelevant.

The number of fruits per plant ranged from 0.5 (OFI-14) to 8.5 ("Bianca" cultivar), and from 11 (OFI-14) to 68 ("Bianca" cultivar) in years 2 and 3, respectively (Fig. 3.3). Statistically significant differences were found among populations for the number of fruits per plant (Table 3.3). In the third year after planting, the Games–Howell post hoc test indicated significant differences among the OFI populations. The group including the "Gialla" and "Bianca" cultivars and the OFI-08. OFI-15 and OFI-19 ecotypes, had a higher number of fruits per plant than the remaining populations.

The 'Gialla' and 'Bianca' cultivars had the highest production of fruit per plant, with 5.2 and 5.1 kg produced in the third year after plantation, respectively (Fig. 3.3). In the group with the 16 OFI ecotypes, the lowest values of fruit production per plant were found in the OFI-03 (0.9 kg $plant^{-1}$) and OFI-14 (1.0 kg $plant^{-1}$), and the highest values were found in the OFI-08 (3.8 kg $plant^{-1}$) and OFI-13 (2.9 kg $plant^{-1}$). The one-way ANOVA revealed the existence of significant differences among the 18 populations for the production of fruit per plant (Table 3.3). The Games–Howell post hoc test showed significant differences among the OFI populations. The group constituted by the two improved cultivars ("Gialla" and "Bianca") and the OFI-08 ecotype did not differ significantly and outperformed the remaining populations concerning the fruit production per plant.

The distribution of the fruit across two size categories of fresh weight, in the third year after plantation, is displayed in Fig. 3.4. The populations with orange pulp fruits (OFI-5, OFI-12, OFI-13, OFI-14, OFI-20 and the "Gialla" cultivar) had larger fruits than the populations with white pulp fruits (OFI-01, OFI-03, OFI-8, OFI-09, OFI-11, OFI-15, OFI-16, OFI-17, OFI-18,

OFI-19 and the “Bianca” cultivar). In the latter group, more than 60% of fruits had a fresh weight lower than 80 g.

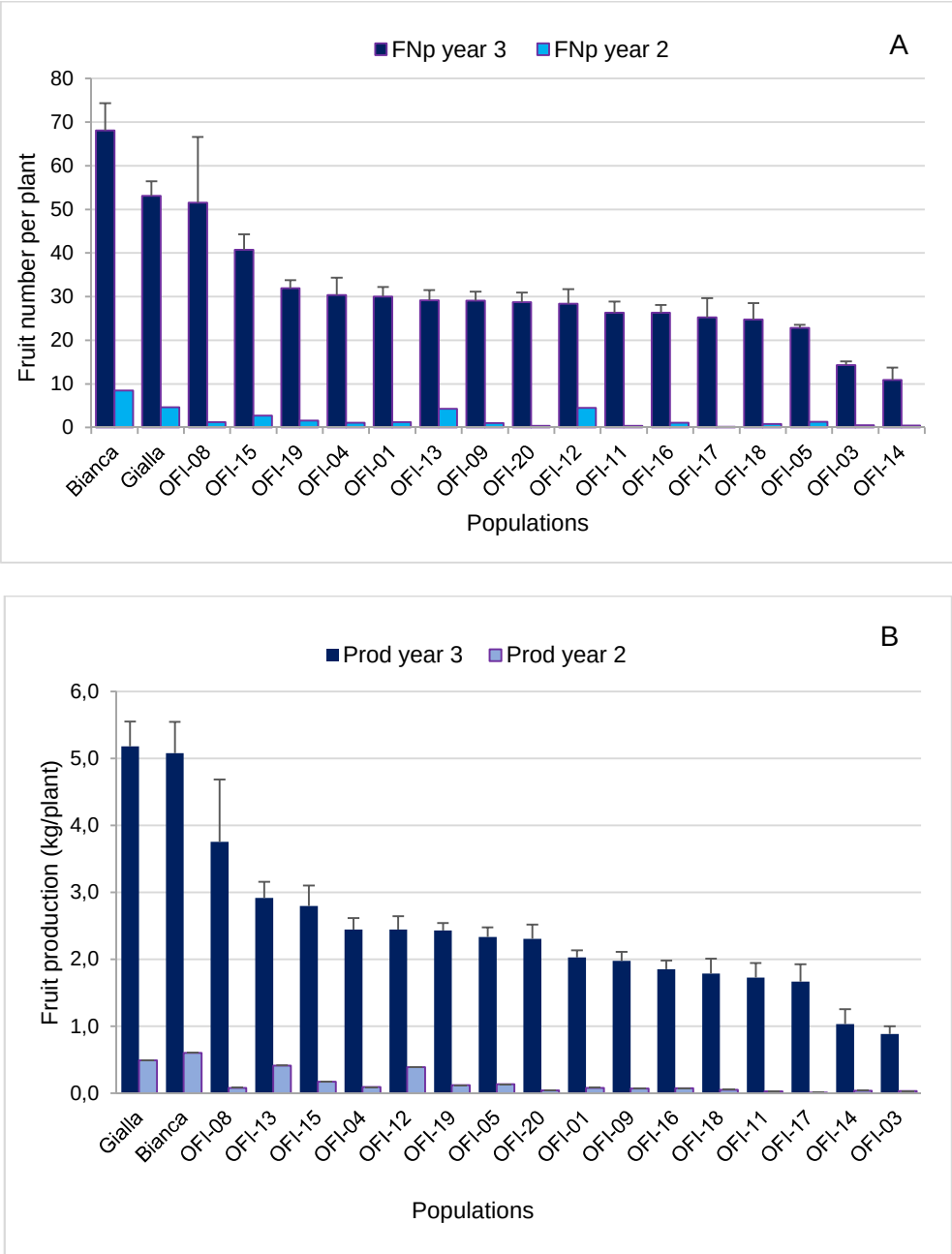

Figure 3.3 Number of fruits per plant (A) and fruit production per plant (B) in the 18 populations of *Opuntia ficus-indica* (OFI) studied, in the second and third year after planting.

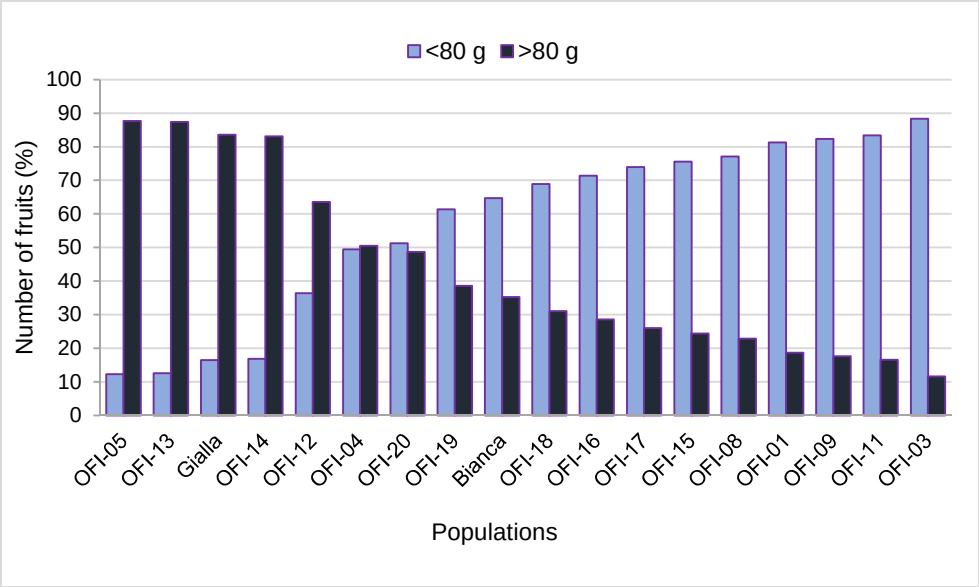

Figure 3.4 Distribution of fruits of *Opuntia ficus-indica* (OFI) populations across two weight categories, according to fresh weight, in the third year after planting.

The Italian "Gialla" and "Bianca" cultivars had the highest fruit yields compared to the Portuguese ecotypes. A fruit production of 5.2 kg plant^{-1} (13.9 Mg ha^{-1} fresh fruit for a density of 2667 plants ha^{-1}, 0.27 plants m^{2}) in the third year after planting was obtained in the "Gialla" cultivar. The observed values were slightly higher than those previously reported by Caloggero and Parera (2004) who obtained a production of 3.7 kg plant^{-1} in the third year after plantation for the "Gialla" cultivar. A fresh weight/dry weight ratio of 6.2 was observed for the fruit in this cultivar, in the current experiment. With regard to the cladode biomass and fruit production, a total DM productivity of 16.13 Mg ha^{-1} was obtained. The ecotype OFI-08 had a fruit production of 10.1 Mg ha^{-1} (3.8 kg plant^{-1}), while the ecotype OFI-13 had the highest production among the ecotypes with orange pulp fruits of 7.73 Mg ha^{-1} (2.9 kg plant^{-1}).

This is the first report of fruit yield and quality measurements from a field trial of Portuguese ecotypes of *O. ficus-indica*. The OFI local ecotypes displayed variability in biomass and fruit production, as well as in the shape of the cladodes, the presence or absence of spines, the corolla colour and the pulp colour.

The main aim of the current study was the evaluation of a group of ecotypes for fruit production in the first years after plantation and the plant density (2 667 plants ha^{-1}) used was higher the one usually used in commercial orchards. In Italy, the plant spacing of the orchards for fruit production ranges from 4 × 6 m (416 plants ha^{-1}) to 5 × 7 m (290 plants ha^{-1}). Usually, the plants begin to yield fruits 2 to 3 years after planting and achieve their maximum production 6 to 8 years after planting (Inglese *et al.*, 2002).

The OFI-08, a white pulp ecotype, had the highest fruit production among the studied ecotypes, however 77% of the total production had a small size and weight and was unsuitable for commercialization. White-flesh fruits are very sensitive to postharvest handling and to specific pests, such as *Ceratitis capitate* (Inglese *et al.,* 2002).

In Italy, the fruits are sorted according to their size and weight. There is a classification scheme, with extra-large fruits being over 160 g, first class being 120–160 g, second class being 80–100 g and third class being below 80 g (Barbera *et al*., 1992). According to Inglese *et al*. (2002), the size of fruit to export must exceed 120 g and should have a minimum 13% sugar content. Under the experimental conditions in the current study, the "Gialla" cultivar and the group of ecotypes with orange pulp produced fruits had a larger size and weight, and in all of them the sugar content was above 13%. In this group, the ecotype OFI-13 was the most promising because the majority (87.4%) of its fruits achieved the minimum size and weight for commercialization. The best-appreciated fruits by international markets have a yellow-orange flesh, such as the "Gialla" cultivar. Fruits with white or greenish flesh are important only for regional or local markets, and their international trade is not relevant (Liguori and Inglese, 2015).

The *Opuntia ficus-indica* is a multifunctional plant, even if cultivated for the main purpose of fruit production. The cladodes from pruning can be used as forage nutrition for ruminants and ultimately cactus pear plantations can contribute to the carbon stock in perennial structures.

A ranking of Portuguese accessions of OFI was made according to the biomass and fruit production per plant. Three OFI ecotypes did not differ significantly from cv. "Gialla" in terms of biomass production. In terms of fruit production, the cultivars "Gialla" and "Bianca" clearly outperformed the Portuguese ecotypes, reflecting their origin as improved plant material. Among the 16 Portuguese OFI populations, a variation in fruit yields and fruit distribution across the weight categories was found. The current study showed that OFI is a valid crop for marginal soils and could be cultivated in a non-tillage system, provided that high yield cultivars and appropriate agronomic practices, i.e. pruning, fruit thinning, fertilization and irrigation are used.

The chemical composition as well sensory attributes are determinant in fruit quality. Information on the sensorial attributes of the cactus pear fruit should be further investigated. Therefore, the constitution, training, and validation of a panel selection should be carried out in order to build the sensory profile of the different cultivars and ecotypes.

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4. Nutritional value of *Opuntia ficus-indica* cladodes from Portuguese ecotypes

This chapter is based on the following published paper:

Rodrigues, A.M., Pitacas, I.S., **Reis, C.M.G**. & Blasco-Ruiz, M. (2016). Nutritional value of *Opuntia ficus-indica* cladodes from Portuguese ecotypes. *Bulgarian Journal of Agricultural Science*, 22(1), 40-45. http://www.agrojournal.org/22/01-07.pdf

4.1 Summary

The use of *Opuntia ficus-indica* cladodes as forage for ruminants has been very important in the semi-arid and arid regions of the world. *Opuntia ficus-indica* cladodes can be fed to small ruminants especially during periods of the year characterized by low quality and quantity of pasture. In Mediterranean regions, such as South Portugal, quantity and quality of pasture are satisfactory during the rainy season. However, in critical times of the year, the shortage and low nutritive value of forage decreases ruminant milk and meat production. The aim of this study was to evaluate the nutritional profile of cladodes from five different spineless Portuguese ecotypes of O. *ficus-indica* compared with the "Gialla" cultivar and to evaluate their potential use as a feed for ruminants. Among populations' significant differences were found in crude protein and ash content, and different groups were unfolded. In general, *O. ficus-indica* had low contents of dry matter (DM), crude protein (CP) and neutral detergent fibre (NDF) and high contents of non-fibre carbohydrates (NFC) and metabolizable energy (ME). Given the importance that DM, CP and NDF have for ruminant nutrition and feeding, we conclude that *O. ficus-indica* can be used to feed small ruminants provided that animals have access to dry forage and a feed source with a high CP content. Used as fodder, *O. ficus-indica* seems to be an interesting feed option for small ruminants in the driest period of the year.

4.2 Introduction

Opuntia ficus-indica (L.) Miller is a species from the Cactaceae family with a centre of origin and domestication in Mexico. It is widely distributed in other regions of the world, such as Africa, Australia and the Mediterranean basin, where it grows in the most diverse ecological conditions (Sáenz *et al.*, 2006).

Introduction of this species into the Iberian Peninsula probably occurred at the end of the 15th century, after the discovery of America, spreading throughout the Mediterranean basin (Le Houerou, 1996; Kiesling, 1998; Anderson, 2001). In Portugal, *O. ficus-indica* exhibits ruderal behaviour and is usually found at the edges of roads and paths. Cultivated for its edible fruits and as a hedge plant, this species is widely naturalized (Reis *et al.*, 2014).

The two forms of the species, *O. ficus-indica* f. *inermis* Weber and *O. ficus-indica* f. *amyclaea* (Ten.) Schelle, are found in Portugal, as well as intermediate types according to spine presence. In Portugal, as in other Mediterranean countries, inland areas are under severe drought, particularly during extensive summers, and global warming is expected to deeply affect these areas in the near future. Due to its morpho-physiological characteristics and multiple economic uses, *O. ficus-indica* represents an alternative crop for these regions

The use of *O. ficus-indica* (OFI) cladodes as forage for ruminants has been very important in semi-arid and arid regions, where long drought periods are common with high summer and low winter temperatures. These factors cause both low forage production and availability. In Mediterranean regions, such as South Portugal, the quantity and quality of pasture is satisfactory during the rainy season. However, in critical times of the year, the shortage and low nutritive value of forage decreases ruminant milk and meat production

The cactus pear can play a stabilizing role in agriculture, as it can prevent stock losses during droughts, save natural grasslands from overgrazing, increase farm income and alleviate poverty in rural areas. Although it has been considered to be poor in terms of crude fibre and crude protein, *O. ficus-indica* is considered to be rich in *in-vitro* digestibility and water content and is often the only source of green forage in the dry season (Silva and Santos, 2007). Additionally, the spineless *O. ficus-indica* plants have good palatability (Ben Salem and Ennouri, 2013).

A common strategy used for improving ruminant performance in Mediterranean and semiarid regions is adequate feed management during periods of scarce forage. In some studies, cactus pears have been used as forage to feed sheep (Ben Salem and Smith, 2008; Rekik *et al.*, 2010; Costa *et al.*, 2012), dairy goats (Costa *et al.*, 2009; Andrade-Montemayor *et al.*, 2011) and dairy cows (Silva and Santos, 2007; Vilela *et al.*, 2010).

Other authors have evaluated cactus pear supplementation and its contribution as a source of water for sheep (Tegegne *et al.*, 2007) and dairy goats (Costa *et al.*, 2009).

There is a lack of information regarding the nutritional value of cladodes from different Portuguese ecotypes of *O. ficus-indica*. The aim of this study was to evaluate the nutritional profile of these cladodes compared with the "Gialla" cultivar (cv.) and to evaluate their potential use as a feed for ruminants. The results were compared among the studied populations and with previous studies.

4.3 Materials and Methods

Plant material and experimental design

On April 2012, mature OFI cladodes were collected from fifteen individuals of five different ecotypes growing in the central and southern regions of Portugal (Table 4.1). The fifteen cladodes of each ecotype were single planted in May 2012 at the Scholl of Agriculture of Castelo Branco, Portugal (39º 49' 17.00'' N; 7º 27' 41.00'' W, elev. 365 m). The "Gialla" cv. was included for comparison. All of the plants were spineless cacti (*O. ficus-indica*).

Table 4.1 Identification of the *O. ficus-indica* populations used in the study of the nutritional value of the cladodes.

Ecotype/cultivar	Origin	Altitude (m)
OFI-04	Portalegre	372
OFI-05	Arronches	293
OFI-12	Cacela-a-Velha	20
OFI-13	Monforte da Beira	260
OFI-14	Idanha-a-Velha	275
OFI, cv."Gialla"	Italy	------

The experiment consisted of a randomized complete block design with three replicates, each replicate consisting of a row with 5 plants. The plant spacing was 1.5 x 2.5 m (2,667 plants ha^{-1}). The experiment was conducted in a granitic soil type, with pH 6.1 and a low organic matter content. Nitrogen, phosphorus and potassium fertilizers were applied at a rate of 40 kg ha^{-1} each to reduce possible differences in soil fertility. No irrigation and notillage were used. Weeds were controlled by mechanical mowing each three months.

The Köppen-Geiger climate classification for Castelo Branco is Csa. The average annual temperature in Castelo Branco is 15.9 °C. July and August are the driest and warmest months, with average temperatures above 24 ºC. The months of December, January and February are the coldest, with average temperatures below 10 ºC. Approximately 783 mm of precipitation fall annually. In winter, there is much more rainfall than in summer. Most precipitation occurs in December, with an average of 124 mm.

Sampling and laboratory analyses

One sample from each of the three replicates was collected in September 2013 (at the end of the dry season). Each sample was a composite of one-year-cladodes randomly harvested from five individuals of each ecotype. In the laboratory, cladodes were cut into 25 cm^2 pieces using a sharp knife. All of the cladode pieces were cut into halves to facilitate drying and were dried to constant mass in a force draught oven at 65 ºC (± 5 ºC) for 72 hours. After determining moisture, cladode pieces were passed through a laboratory mill with a one millimetre sieve. The dried plant material was stored in tightly sealed plastic bottles until further chemical analysis.

Each cladode sample was analysed for total dry matter (DM), total ash (Ash), crude protein (CP) and ether extract (EE) according to AOAC (2000), and for neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) according to the procedures described by Van Soest *et al.* (1991). The CP was calculated by multiplying the percent nitrogen by a factor of 6.25 (Ruddell *et al.*, 2002).

Hemicellulose was determined by subtracting NDF – ADF, and cellulose was determined by subtracting ADF – ADL. Non fibre carbohydrates (NFC) were calculated by difference whereby the sum of the percentages of CP, EE, Ash and NDF were subtracted from 1000 [NFC (g kg^{-1} DM) = 1000 – (CP + EE + Ash + NDF)] (NRC, 2001).

Total digestible nutrients (TDN) were calculated with the prediction equation described by Bath and Marble (1989) cited by Coppock (1997) [TDN% = 82.38 – (0.7515 × ADF%)]. Metabolizable energy (ME) for ruminants was calculated with the prediction equation proposed by NRC (2007) [ME (MJ kg^{-1} DM) = (TDN% × 3.6)/100 × 4.184]. Compared with the original equation, this formula includes a DM × 4.184 factor that accounts for the conversion of ME data from Mcal to MJ.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the General Linear Model available in IBM SPSS version 21 (IBM Corp., Armonk, NY, USA), and the Tukey test was used to detect significant differences ($P < 0.05$) among treatment means.

4.4 Results and discussion

The average nutritional value of the cladodes from the different *O. ficus-indica* populations studied is presented in Table 4.2.

Table 4.2 Nutritional value on a dry matter basis of the *Opuntia ficus-indica* cladodes from the different populations studied.

Nutritional parameters	Populations						
	OFI-04	OFI-05	OFI-12	OFI-13	OFI-14	cv. "Gialla"	Total
DM (%)	12.85 ±1.62	14.58 ±1.14	14.10 ±0.67	13.03 ±0.86	13.74 ±0.86	14.17 ±1.43	13.75^{ns} ±1.24
ME (MJ kg^{-1} DM)	11.16 ±0.20	11.26 ±0.42	11.27 ±0.08	11.17 ±0.15	11.38 ±0.16	11.24 ±0.13	11.24^{ns} ±0.22
TDN (%)	73.79 ±1.324	74.41 ±2.78	74.48 ±0.56	73.83 ±1.01	75.20 ±1.08	74.31 ±0.83	74.34^{ns} ±1.43
CP (g kg^{-1} DM)	69.94^{b} ±1.13	72.57^{ab} ±7.37	82.52^{a} ±9.55	78.44^{ab} ±7.74	68.01^{b} ±5.11	72.45^{ab} ±8.11	73.99 ±8.26
EE (g kg^{-1} DM)	15.71 ±1.24	15.65 ±2.95	14.43 ±1.01	13.58 ±1.91	14.10 ±1.51	14.70 ±0.62	14.70^{ns} ±1.77
NDF (g kg^{-1} DM)	198.99 ±13.35	183.85 ±37.66	186.05 ±28.90	198.05 ±31.75	164.67 ±16.12	179.30 ±13.23	185.15^{ns} ±26.33
Hem (g kg^{-1} DM)	84.65 ±18.94	77.78 ±4.33	80.97 ±24.50	84.26 ±21.54	69.19 ±17.89	71.85 ±13.98	78.12^{ns} ±17.76
ADF (g kg^{-1} DM)	114.35 ±17.62	106.06 ±36.97	105.08 ±7.39	113.79 ±13.43	95.49 ±14.32	107.45 ±11.00	107.04^{ns} ±18.99
Cel (g kg^{-1} DM)	105.82 ±19.65	96.83 ±32.06	94.30 ±6.03	105.54 ±12.04	88.33 ±11.81	98.33 ±9.83	98.19^{ns} ±17.33
ADL (g kg^{-1} DM)	8.52 ±2.49	9.24 ±5.13	10.79 ±2.69	8.25 ±1.89	7.16 ±2.65	9.12 ±2.09	8.85^{ns} ±3.01
NFC (g kg^{-1} DM)	629.63 ±23.32	641.77 ±42.32	636.87 ±19.95	612.38 ±50.98	665.58 ±13.05	641.70 ±15.55	637.99^{ns} ±32.87
Ash (g kg^{-1} DM)	85.73^{ab} ±11.57	86.17^{ab} ±5.86	80.12^{b} ±4.98	97.55^{a} ±11.48	87.63^{ab} ±5.17	91.85^{ab} ±8.25	88.18 ±9.49

$^{a\,b}$ – Means with different superscripts in the same line differ significantly ($P<0.05$); values are means ± standard deviation; ns –$P>0.05$; DM – dry matter; ME – metabolizable energy; TDN – total digestible nutrients; CP – crude protein; EE – ether extract; NDF – neutral detergent fibre; Hem – hemicellulose; ADF – acid detergent fibre; Cel – cellulose; ADL – acid detergent lignin; NFC – non fibre carbohydrates

No statistically significant difference was found among the DM values of the different samples. Considering the DM values reported for cacti by NRC (2007) (26%) and Cordova-Torres *et al.* (2009) (15.5 to 16.5%), the average DM content of the different OFI populations studied (13.75% ± 1.24) was low. The highest DM content (14.58% ± 1.14) was observed in the OFI-05 ecotype, and the lowest DM content was observed in the OFI-04 ecotype (12.85% ± 1.62). However, our results were higher than those reported by Mciteka (2008) (9.13% DM), Fuentes-Rodriguez (1997) (11.3% DM), Rekik *et al.* (2010) (9.7% DM) and Tegegne *et al.* (2007) (12.2% DM) and were within the range of values reported by Andrade-Montemayor (2011) (8 to 15% DM), Silva and Santos (2007) (7.62 to 14.4% DM) and Costa *et al.* (2009) (10 to 14% DM). The differences may be explained by variations in the age of the analysed

cladodes, the harvest time, and genotype-level variations. Dry matter content tends to increase with the age of the cladode (Tegegne, 2001). The high water content of *Opuntia* cladodes makes them a bulky feed and therefore difficult to transport over long distances. However, the high water content in cactus pears represents an important alternative to satisfy the water requirements of animals in arid and semi-arid regions, where water may be a limiting factor for animal production. The water intake of dairy goats was markedly reduced when cactus pears were part of their diet (Costa *et al.*, 2009). Nefzaoui and Ben Salem (1998) showed that water intake from freely available water sources was zero when the daily cladode consumption by sheep was approximately 300 g of dry matter.

There were no significant differences ($P > 0.05$) in ME and TDN contents among the different OFI ecotypes and the "Gialla" cv. (Table 4.2). The ME content varied between 11.16 MJ kg^{-1} DM (± 0.20) (OFI-04 ecotype) and 11.38 MJ kg^{-1} DM (± 0.16) (OFI-14 ecotype), and the TDN content varied between 73.79% (± 1.324) (OFI-04 ecotype) and 75.20% (± 1.08) (OFI-14 ecotype). Our results for ME were higher than those reported by NRC (2007) (9.62 MJ kg^{-1} DM) and Costa *et al.* (2012) (9.2 MJ kg^{-1} DM); our results for TDN were also higher than the reported range of 60.8%-68.6% (NRC, 2007; Vilela *et al.*, 2010; Costa *et al.*, 2012).

The OFI populations studied showed statistically significant differences in CP content ($P < 0.05$), which varied between 68.01 g kg^{-1} DM (± 5.11) (OFI-14 ecotype) and 82.52 g kg^{-1} DM (± 9.55) (OFI-12 ecotype) (Table 4.2).

The CP content of the "Gialla" cv. was 72.45 g kg^{-1} DM (± 8.11). The average CP content of the different OFI populations analysed (73.99 g kg^{-1} DM ± 8.26) was higher than that reported by Magalhães (2004) (51.4 g kg^{-1} DM), NRC (2007) (50.0 g kg^{-1} DM), Tegegne *et al.* (2007) (50.6 g kg^{-1} DM), Mciteka (2008) (55.4 g kg^{-1} DM), Villegas-Diaz *et al.* (2008) (59.0 g kg^{-1} DM), Abidi *et al.* (2009) (38.0 g kg^{-1} DM), Rekik *et al.* (2010) (44.0 g kg^{-1} DM), and Vilela *et al.* (2010) (44.0 g kg^{-1} DM). These results may be due to the age of the cladodes used in our study (one year old cladodes) or to differences in soil nitrogen availability. Teles *et al.* (1997) reported a CP content of 110.3 g kg^{-1} DM, a much higher value than ours. According to Ben Salem and Smith (2008), increasing the CP content of OFI cladodes used to feed animals should be considered in breeding programs. Nitrogen fertilization is another strategy to reach the latter objective.

The average EE content of the different OFI ecotypes and the "Gialla" cv. was 14.70 g kg^{-1} DM (± 1.77), and it varied between 13.58 g kg^{-1} DM (±1.91) (OFI-13 ecotype) and 15.71 g kg^{-1} DM (± 1.24) (OFI-04 ecotype). Several authors reported EE values between 21 g kg^{-1} DM and 23 g kg^{-1} DM (NRC, 2007; Mciteka, 2008; Vilela *et al.*, 2010), which were higher than those determined by us and by Tegegne *et al.* (2007) (11.9 g kg^{-1} DM).

The NDF content varied from 164.67 g kg^{-1} DM (± 16.12) to 198.99 g kg^{-1} DM (± 13.35) for the ecotypes OFI-14 and OFI-04, respectively (Table 4.2). The average NDF content of the different OFI populations analysed (185.15 g kg^{-1} DM ± 26.33) was lower than that reported by NRC (2007) (290.0 g kg^{-1} DM), Tegegne *et al.* (2007) (238.8 g kg^{-1} DM), Vilela *et al.* (2010) (314.0 g kg^{-1} DM), Abidi *et al.* (2009) (251 g kg^{-1} DM), Rekik *et al.* (2010) (306 g kg^{-1} DM), Villegas-Diaz *et al.* (2008) (435 g kg^{-1} DM), Andrade-Montemayor *et al.* (2011) (460 g kg^{-1} DM), and Costa *et al.* (2012) (312 g kg^{-1} DM). This difference may be related to the young age of the cladodes used in the present study, which have lower NDF contents in the cell wall.

The ADF content of the different OFI cladodes varied between 95.49 g kg^{-1} DM (± 14.32) (OFI-14 ecotype) and 114.35 g kg^{-1} DM (± 17.62) (OFI-04 ecotype) (Table 4.2). Some authors reported higher ADF values of *O. ficus-indica* ranging between 136.6 g kg^{-1} DM and 287 g kg^{-1} DM (Magalhães, 2004; NRC, 2007; Tegegne *et al.*, 2007; Mciteka, 2008; Villegas-Diaz *et al.*, 2008; Cordova-Torres *et al.*, 2009; Vilela *et al.*, 2010; Andrade-Montemayor *et al.*, 2011; Costa *et al.*, 2012). Both the NDF and ADF contents indicate that O. *ficus-indica* cladodes cannot be regarded as a sole roughage source.

The average hemicellulose content in the present study was 78.12 g kg^{-1} DM (± 17.76) (Table 4.2). The hemicellulose content varied from 69.19 g kg^{-1} DM (± 17.89) (ecotype OFI-14) to 84.65 (± 18.94) (ecotype OFI-04). These results were lower than the value reported by Costa *et al.* (2012) (95 g kg^{-1} DM) and higher than the value reported by NRC (2007) (60.0 g kg^{-1} DM). The average cellulose content (98.19 g kg^{-1} DM ± 17.33) was lower than 131.8 g kg^{-1} DM (Tegegne *et al.*, 2007) and 123 g kg^{-1} DM (Vilela *et al.*, 2010). The cellulose content of the different OFI populations varied between 88.33 g kg^{-1} DM (± 11.81) (OFI-14 ecotype) and 105.82 g kg^{-1} DM (± 19.65) (OFI-04 ecotype) (Table 4.2).

The highest ADL content was recorded for the OFI-12 ecotype (10.79 g kg^{-1} DM ± 2.69) and the lowest for the OFI-14 ecotype (7.16 g kg^{-1} DM ± 2.65) (Table 4.2). Our results were lower than the values reported by Tegegne *et al.* (2007) (30.6 g kg^{-1} DM) and Vilela *et al.* (2010) (32 g kg^{-1} DM).

The NFC content varied from 612.38 g kg^{-1} DM (± 50.98) (OFI-13 ecotype) to 665.58 g kg^{-1} DM (± 13.05) (OFI-14 ecotype) (Table 4.2). These results were higher than the values reported in other studies, which varied between 469 g kg^{-1} DM and 530 g kg^{-1} DM (Magalhães, 2004; NRC, 2007; Tegegne *et al.*, 2007; Vilela *et al.*, 2010; Costa *et al.*, 2012).

There were significant differences ($P < 0.05$) in ash content among the different OFI populations (Table 4.2). The ash content varied between 80.12 g kg^{-1} DM (± 4.98) and 97.55 g kg^{-1} DM (± 11.48). Several authors reported that the ash content of *Opuntia* cladodes may vary between 131 g kg^{-1} DM and 255 g kg^{-1} DM (Fuentes-Rodriguez, 1997; NRC, 2007; Tegegne *et al.*, 2007; Mciteka, 2008; Andrade-Montemayor *et al.*, 2011).

Analysing the obtained results we conclude that among the studied Portuguese populations of *O. ficus-indica*, the OFI-12 ecotype is the most suitable for feeding ruminants. The cladodes of this ecotype are spineless and have higher CP levels compared with the other Portuguese ecotypes as well as the "Gialla" cv. In general, *O. ficus-indica* had low contents of DM, CP and NDF and high contents of NFC and ME. Given the importance that DM, CP and NDF have for ruminant nutrition and feeding, we conclude that *O. ficus-indica* can be used to feed small ruminants provided that animals have access to dry forage and a feed source with a high CP content

Used as fodder, *O. ficus-indica* seems to be an interesting feed option for small ruminants in the driest period of the year, when there is low quality and quantity of pasture. If the main purpose of cladode production is roughage for ruminant feeding, potential breeding programs should focus on the OFI-12 ecotype and its CP and NDF contents. For regions were the main purpose of cacti growth is fruit production, cactus forage may be an important by-product of pruning that can help feed livestock.

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5. Bioactive compounds and morphology in *Opuntia* spp. fruits from Portuguese ecotypes

This chapter is based on the following published paper:

Reis, C.M.G., Gouveia, C., Vitorino, M.C., Gazarini, L.C., Ribeiro, M.M. & Peres, F. (2017). Bioactive compounds and morphology in *Opuntia* spp. fruits from Portuguese ecotypes. *Bulgarian Journal of Agricultural Science,* 23(6), 929–938. http://www.agrojournal.org/23/06-06.pdf.

5.1 Summary

The *Opuntia* spp. has minimal soil and water requirements, and the *O. ficus-indica*, in particular is sought to be an alternative for the Mediterranean region agricultural economy. The morphology, bioactive compounds and antioxidant properties of fruits were studied in twenty Portuguese ecotypes belonging to four *Opuntia* species (*O. ficus-indica*, *O. robusta*, *O. dillenii* and *O. elata*). The ecotypes were compared with the *O. ficus-indica* cultivars "Bianca", "Gialla" and "Rossa". The fruits from *Opuntia* spp. ecotypes displayed variability in morphological and bioactive characteristics. The *O. dillenii* ecotypes had the highest betalain content, total phenolic compounds, and antioxidant activity, while *O. elata* had the highest ascorbic acid content. Both *O. dillenii* and *O. elata* had the highest acidity values. The red pulp cv. "Rossa" had the highest betalain content among the *O. ficus-indica* populations, followed by the orange and white pulp ecotypes. The highest amount of total phenolic compounds was found in the white pulp *O. ficus-indica* ecotypes. The ecotype OFI-04 was distinguishable from the others due to the quantitative and qualitative characteristics of its fruit, and it could be considered a new variety. The *Opuntia ficus-indica* orange pulp fruits were larger, heavier and had a higher percentage of pulp as well as a lower percentage of seeds compared to the white pulp fruits. However, the weight of 100 seeds was lower in the white pulp ecotypes. The hierarchical clustering analysis revealed that the ecotypes could be grouped into four major groups, and geographical origin was unrelated to the clustering pattern. This study provides original data on the morphology and bioactive compounds of *Opuntia* spp. fruits from Portuguese ecotypes. The *Opuntia*. spp. is an interesting source of phenolic compounds, betalains, and ascorbic acid, and the moderate consumption of prickly fresh fruit can provide important antioxidant intake.

5.2 Introduction

The genus *Opuntia* (Cactaceae) is native to Central America and was likely introduced in the Iberian Peninsula after the discovery of the Americas between the end of the 15th and beginning of the 16th centuries, later spreading throughout the Mediterranean basin (Barbera *et al*., 1992; Anderson, 2001). The Mediterranean region, particularly inland areas, is currently suffering from severe drought during extensive summers, and global climate change is expected to deeply affect this area in the near future with climatic models predicting drier climates with shorter and wetter rainy seasons followed by long dry summers (Schröter *et al*., 2005). *Opuntia* spp. have minimal soil and water requirements, and *Opuntia ficus-indica* (OFI), in particular, is thought to be an alternative species for the agricultural economy of the Mediterranean region (Barbera *et al*., 1992).

In Portugal, several *Opuntia* species were naturalized (*O. ficus-indica* (L.) Miller, *O. dillenii* (Ker-Gael) Haw., *O*. *robusta* Wendl and *O. elata* Salm-Dick), and the most widespread and economically relevant sp. is both forms of OFI, including the inermis typical form, *Opuntia ficus-indica* f. *ficus-indica* (L.) Miller, and the spiny form, *Opuntia ficus-indica* f. *amyclaea* (Ten.) Schelle (Kiesling, 1998). The OFI local ecotypes have variable plant vigour and biomass production (Reis *et al*., 2018) as well as differences in the shape of the cladodes, presence or absence of spines, spine length, corolla colour, pulp colour, and fruit ripening time (our unpublished results). Traditionally, OFI is cultivated for edible fresh fruit production and hedge establishment under non-irrigated conditions, but recently some farmers have been focusing on OFI orchards that are drip irrigated for fresh fruit production along with a plant layout and spacing design. Cactus pear fruit is a fleshy berry derived from an inferior ovary (acrosarcum) that varies in shape, size, and number of hard seeds, with high total soluble solids content (TSS) (12 to 17%), and low acidity content (0.03 to 0.12% citric acid) (Yahia and Mondragón-Jacobo, 2011). The *O. dillenii* fruits are a source of betacyanin pigments that can be used as a red-purple food colorant and an alternative to beetroot red (Cejudo-Bastante *et al*., 2015). The *O. elata* is cultivated as ornamental plant because the cladodes have few spines, the purple fruits are small, and they can be found growing sub-spontaneously in some places in the centre and north inland regions of Portugal, especially the Douro valley region.

Recently, significant attention has been given to the cactus pear due its nutritional and health benefits as well as its high bioactive antioxidant compound content, including betalains, phenolic compounds, ascorbic acid and carotenoids (Sumaya-Martínez *et al*., 2011; Yahia and Mondragón-Jacobo, 2011; El-Mostafa *et al*., 2014; Albano *et al*., 2015). Additionally, health benefits, such as antioxidant, neuroprotective, anti-inflammatory,

hypoglycaemic and anticancer effects were reported (Tesoriere *et al.,* 2004; Chavez-Santoscoy *et al*., 2009; Serra *et al*., 2013; Jiménez-Aguilar *et al*., 2014).

The information about bioactive antioxidant compounds in Portuguese ecotypes of OFI, *O. robusta* and *O. dillenii* is scarce, and to the best of our knowledge, there are no studies on the composition of *O*. *elata* fruit. The main objectives of this study were i) to characterize the morphology, bioactive compounds and antioxidant properties of *Opuntia* spp. fruits to determine their nutraceutical potential, ii) to classify different species and ecotypes into distinct groups according to their morphology and fruit chemical characteristics using a multivariate analysis approach.

5.3 Materials and methods

Plant material and provenance trial

Cladodes from OFI, *O. elata* and *O. robusta* were planted in a provenance trial at the Castelo Branco School of Agriculture, Portugal (39°49'17"N; 7°27'41"W, elev. 365 m), in 2012. Sixteen OFI Portuguese ecotypes and two improved Italian cultivars (cv.), "Bianca" and "Gialla", which were included for comparison purposes, were studied. The *O. robusta* and *O. elata* were represented by only one ecotype (Table 5.1). The experimental design was a randomized complete block design with three replicates per ecotype, and each replicate had five plants in a row. The provenance trial was planted in a granitic soil with pH 5.9 and low organic matter content, which is a marginal soil with a reduced overall soil profile depth and low water holding capacity. Fertilizers with nitrogen, phosphorus, and potassium were applied at 40 kg ha^{-1} for each element annually to reduce the possible differences in soil fertility. Irrigation (60 mm) was applied in the second and third year of cultivation. No tilling was used, and the weeds were controlled by mechanical mowing. Pruning was performed in spring to lighten the canopy. In the third year after planting, thinning was carried out after flowering to achieve a maximum of 6 fruits per cladode. Afterwards, three samples of 10 full mature fruits were collected from each replicate for each of the different *Opuntia* species established in the provenance trial (Table 5.1). Additionally, two ecotypes of *O. dillenii* were studied, and 30 fruits from each ecotype were collected in fifteen plants from local origin (Table 5.1). Commercially mature fruits from the OFI cv. "Rossa" were acquired from a local producer. In both cases, the fruits were divided into three samples of 10 fruits each.

Table 5.1 Identification, fruit shape, pulp colour and origin of the studied *Opuntia* spp. populations.

Population	Fruit Shape	Pulp colour	Origin	Altitude (m)	Geographic coordinates	
					Latitude	Longitude
OFI-01	Ell	White	Alcochete	25	38°43'32.14"N	8°57'58.22"W
OFI-03	Ell	White	Cascais	185	38°45'23.18"N	9°27'38.48"W
OFI-04	Ovd	Pale yellow	Portalegre	372	39°16'22.45"N	7°26'13.12"W
OFI-05	Ovd	Orange	Arronches	293	39°5'21.06"N	7°12'7.05"W
OFI-08	Ell	White	Melides	29	38°8'28.91"N	8°44'14.28"W
OFI-09	Ell	White	Santo André	25	38°4'38.13"N	8°46'38.08"W
OFI-11	Ell	White	Albufeira	61	37°5'23.33"N	8°17'27.03"W
OFI-12	Ovd	Orange	Cacela-a-Velha	20	37°9'22.50"N	7°32'47.98"W
OFI-13	Ovd	Orange	Monforte da Beira	260	39°45'8.34"N	7°16'54.83"W
OFI-14	Ovd	Orange	Idanha-a-Velha	275	39°59'57.30"N	7° 9'3.51"W
OFI-15	Ell	White	Ponte de Sor	125	39°16'15.45"N	8° 0'44.72"W
OFI-16	Ell	White	Coruche	76	38°54'40.93"N	8°37'17.00"W
OFI-17	Ell	White	Castelo Branco	402	39°48'58.84"N	7°29'37.85"W
OFI-18	Ell	White	Reg. Monsaraz	223	38°27'27.04"N	7°39'21.77"W
OFI-19	Ell	White	Alvega	105	39°27'15.96"N	8° 3'51.88"W
OFI-20	Ovd	Orange	Madeira	116	32°38'54.18"N	16°57'46.38"W
OFI, cv. “Bianca”	Ell	White	Italy	--	--	--
OFI, cv. “Gialla”	Ovd	Orange	Italy	--	--	--
OFI, cv. “Rossa”	Ell	Red	Italy	--	--	--
O. robusta	Rou	Red	Castelo Branco	365	39°49'17.00”N	7°27'41.00”W
O. dillenii, OD-1	Ell	Purple	Lagos	48	37°8’42.24”N	8°40’33.42”W
O. dillenii, OD-2	Ell	Purple	Cacela-a-Velha	20	37° 9'22.50"N	7°32'47.98"W
O. elata	Obl	Purple	S. J. Pesqueira	450	41°9’5.83”N	7°22’5.43”W

OFI – *Opuntia ficus-indica*; OD – *Opuntia dillenii*. Ell – elliptic; Obl – oblong; Ovd – ovoid; Rou – round.

Fruit morphological characterization

The following morphological characteristics were evaluated in three replicates of 10 fruits each: weight (g), length (cm), diameter (cm), shape (measured by the ratio diameter/length), pulp weight (g) and pulp as fruit weight percentage. The seed weight per fruit (g), seed as pulp weight percentage and 100 seed weight (g) were measured in triplicate from pooled samples of ten fruits.

Fruit sample preparation and chemical reagents

The peel was manually separated from the pulp, followed by weighing the pulp and briefly homogenizing it in a kitchen-type blender. Afterwards, the pulp was separated from the seeds, portioned and stored at −80°C until analysis. For *O. elata* fruits, due to the low pulp yield, the whole fruit was used after seed removal. After defrosting, the juice was centrifuged at 14000 rpm for 10 min, and the supernatant was used for pH, acidity, and total soluble solid (TSS, %) determination. The remaining supernatant was filtered through Whatman® filter paper, Grade 42, and the filtrate was used to estimate the total phenolic compound (TPC), the ascorbic acid (AA) and the betalain content (betaxanthins and betacyanins). The

antioxidant activity was quantified after an additional filtration through a 45 µm syringe filter. The readings were collected in triplicate for each sample. All reagents were ACS grade and were purchased from Sigma-Aldrich Company.

TSS, pH, acidity and colour determination

The TSS concentration (%) was determined in the juice using a digital refractometer (Hanna, HI 96800). Total acidity was determined using a pH metre (Radiometer PHM 61) after titration of 10 mL of seedless pulp-juice against 0.01 N NaOH to the end point (pH 8.2), and the results were expressed as a percentage of citric acid. The chromatic characteristics, which were defined by the colorimetric or chromaticity coordinates, the lightness (*L**, ranging from 0, black, to 100, white), the *a** (which takes positive values for reddish colours and negative values for greenish ones), and the *b** (positive for yellowish colours and negative for bluish ones), were determined using a Minolta CR-300 colorimeter.

Ascorbic acid determination

The AA content was determined by UV/Vis spectrophotometry as described in previous studies (Dürüst *et al.*, 1997). Briefly, 0.25 mL of the juice (a different dilution factor in acid oxalic at 0.4% was used from each sample) was added into 0.25 mL of acetate buffer, and 2.0 mL of DCPI (2,6-dichloroindophenol sodium) was added. The absorbance of the mixture was measured immediately at 520 nm using a Biochrom Libra S21 single beam spectrophotometer. Ascorbic acid was used as a reference standard, and the results were expressed as mg ascorbic acid kg^{-1} fresh weight (FW).

Betalain determination

The aqueous pigment extracts were diluted in water to obtain absorption values of $0.9 < A < 1.0$ at their respective maximum absorption. The betalain content (BC) was calculated by spectrophotometry as described in previous studies (Stintzing *et al*., 2005): BC (mg L^{-1}) = (A x DF x MW x 1000)/(ε x 1), where A is the absorption value at the absorption maximum, DF is the dilution factor and 1 is the path length (1 cm) of the cuvette. For the quantification of betacyanins and betaxanthins, the molecular weights (MW) and the molar extinction coefficients (ε) of betanin (MW = 550 g mol^{-1}; ε = 60 000 L mol^{-1} cm^{-1} in H_2O; λ = 538 nm) and indicaxanthin (MW = 308 g mol^{-1}; ε = 48 000 L mol^{-1} cm^{-1} in H_2O; λ = 480 nm) were applied, respectively. The determination was performed using a Biochrom Libra S21 single beam spectrophotometer.

Total phenolic compounds

The TPC was determined using the Folin-Ciocalteau VIS spectrophotometric method (Singleton *et al.*, 1999), and the absorbance measurement was performed in a Jasco 7800 spectrophotometer. Gallic acid was used as a standard to produce the calibration curve, and TPC were estimated using three average readings and expressed in mg of Gallic acid equivalents (GAE), mg kg^{-1} FW.

DPPH radical scavenging activity assay

The antioxidant capacity of the filtered juices was tested using the DPPH (1,1-diphenyl-2-picrylhyydrazyl) approach (Yen and Duh, 1994). The juice was centrifuged and filtered through a 45 µm syringe filter. A methanol DPPH solution (0.06 mM) was mixed with the juice at different concentrations and then solutions with different concentrations were made. The mixtures were vortexed, incubated for 30 min in the dark and placed in a UV/Vis spectrophotometer (Jasco 7800) where the absorbance was read at 517 nm. The inhibition of free radical DPPH (*I* %) was calculated as $I\% = [(A_0 - A_1)/A_0] \times 100$, where A_0 and A_1 are the absorbance values of the blank (all reagents except the test compounds) and the tested samples, respectively. The *I*% was plotted against the respective concentrations that were used. The linear equation of each graph was used to calculate $IC_{50,}$ which is the antioxidant concentration required to inhibit the DPPH absorbance by half.

Data analysis

The data was analysed using one-way ANOVA or, in absence of variance homogeneity, Welch ANOVA was performed, followed by pairwise comparisons using the Tukey or the Games-Howell post-hoc tests, respectively. Statistical significance was accepted with 5% as the probability of type I error for both the omnibus test and the multiple comparisons test. A principal component analysis (PCA) was conducted using morphological and chemical data, and a hierarchical cluster analysis was performed with standardized data (*Z* scores) using the Euclidean distances following the between-groups (average) linkage method. The decision about the number of clusters to retain was made by plotting the number of clusters on the x-axis against the distance at which objects or clusters were combined on the y-axis (Sarstedt and Moi, 2014). The statistical analyses were performed using IBM SPSS Statistics software v.21 (IBM Corp., NY.).

5.4 Results and discussion

Fruit morphological characterization

The shapes of the fruits were oblong (*O. elata*), elliptic (*O. dillenii* and in the white pulp fruits from OFI), ovoid (in orange pulp fruits from OFI) and round (*O. robusta*) (Fig. 5.1 and Table 5.1).

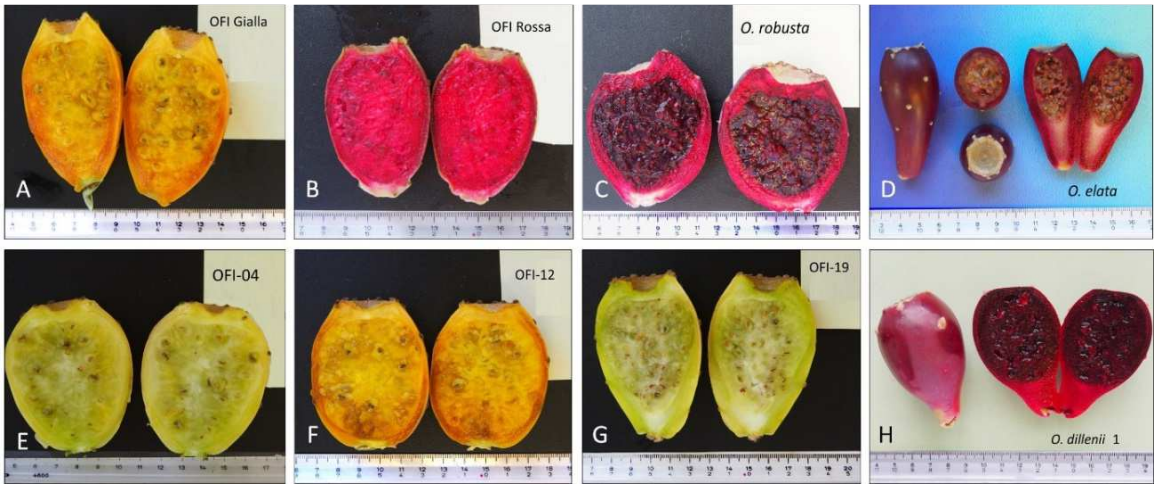

Figure 5.1 Cactus pear fruit from some populations studied. A – *Opuntia ficus-indica* (OFI) cv. "Gialla"; B – OFI cv. "Rossa"; C – *O. robusta*; D – *O. elata*; E – OFI-04; F – OFI-12; G – OFI-19; H – *O. dillenii* (OD-1).

The fruit weight varied between 19.5 (*O. elata*) and 132.5 g (OFI-13) (Fig. 5.2A). In *O. dillenii* (the mean value of the two populations) and *O. robusta*, the mean weights of the fruit were 50.1 and 124.9 g, respectively. The mean weights of the fruits were 90.6 g in the OFI populations with white pulp fruits and 121.4 g in the populations with orange pulp fruits, and significant differences were found, Welch's F (18, 203.9) = 165.2, $p < 0.05$. The width (R^2 = 0.91) and the pulp weight (R^2 = 0.96) had the highest correlation coefficients with the weight of the fruit. The pulp yield varied between 22.6 (*O. elata*) and 67% (OFI cv. "Rossa") (Fig. 5.2A). The OFI mean pulp yield was 53.8 and 64.30% for the white and orange pulp fruits, respectively, Welch's F (18, 203.9) = 136.5, $p < 0.05$. *O. elata* had the smallest fruits, while in the OFI ecotypes the lengths varied from 7.0 (OFI-20) to 8.0 cm (OFI-08), and the diameters varied from 4.8 (OFI-03, OFI-09, OFI-11 and cv. "Rossa") to 5.8 cm (OFI-13 and cv. "Gialla") (Fig. 5.2B). The seed weight per fruit varied between 5.8 (*O. robusta*) and 1.1 g (*O. elata*) (Fig. 5.3A). The amount of seeds as a percentage of pulp weight ranged between 22.8 (*O. elata*) and 3.7% (OFI-04), and the weight of 100 seeds varied between 0.8 (*O. elata*) and 3.1 g (*O. dillenii*) (Fig. 5.3A). In OFI ecotypes, the following average values were

found in the white pulp and orange pulp fruits: seed weights per fruit were 3.0 and 3.7 g, the amount of seeds as pulp weight percentage were 5.9 and 4.6%, and the weights of 100 seeds were 1.7 and 2.1 g, respectively (Fig. 5.3B). The ecotype OFI-04 contrasted the other OFI ecotypes due to its pale yellow pulp, ovoid shape, and low seed weight per fruit as well as the amount of seeds as a percentage of pulp weight. The OFI orange pulp fruits were larger, heavier and had a higher percentage of pulp as well as a lower percentage of seeds compared to the white pulp fruits. However, the weight of 100 seeds was lower in the white pulp ecotypes.

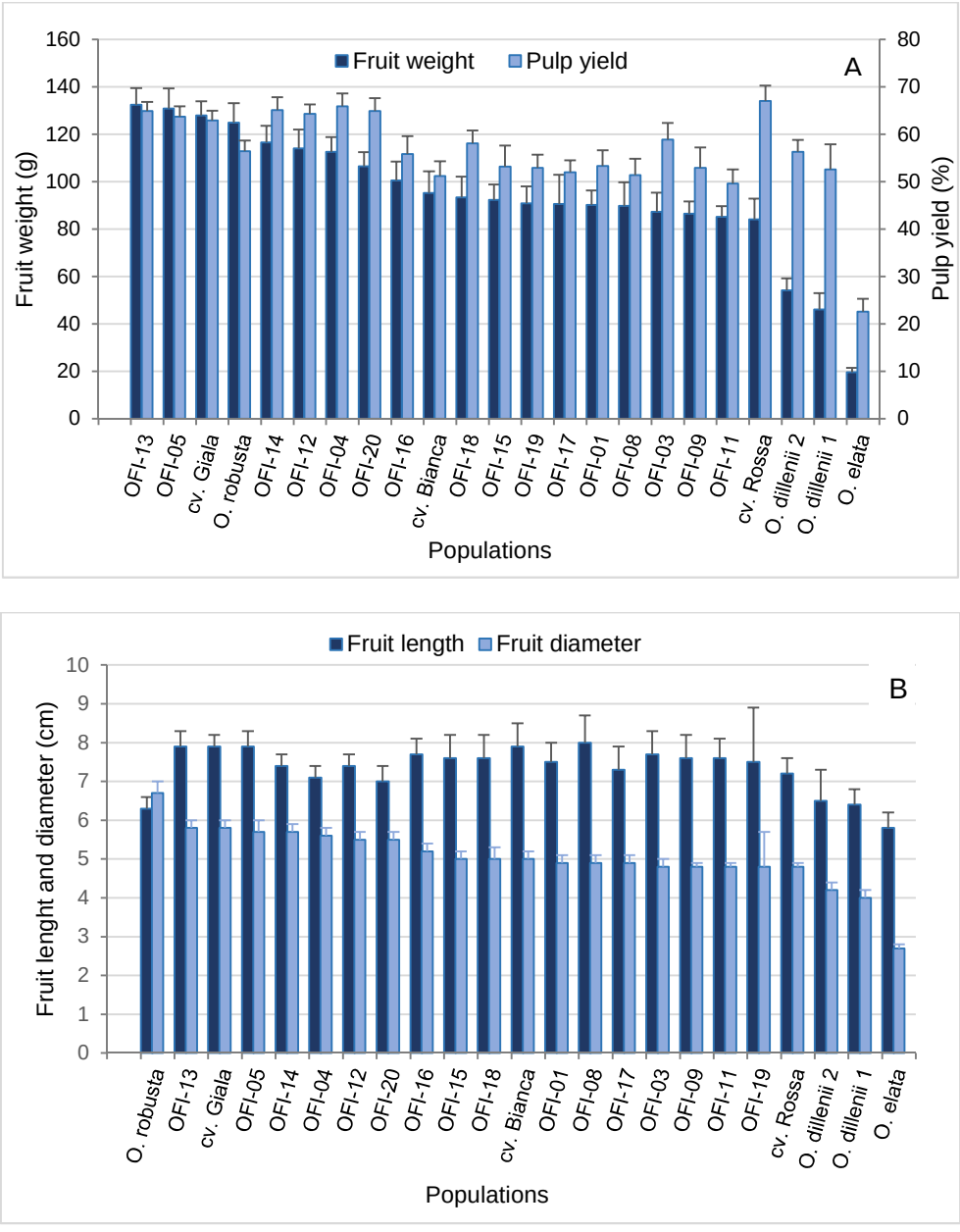

Figure 5.2 A - Fruit weight (g) and pulp yield (%); B - Fruit length (cm) and diameter (cm). Values are the means from the *Opuntia* spp. populations studied (n=30 fruits per population).

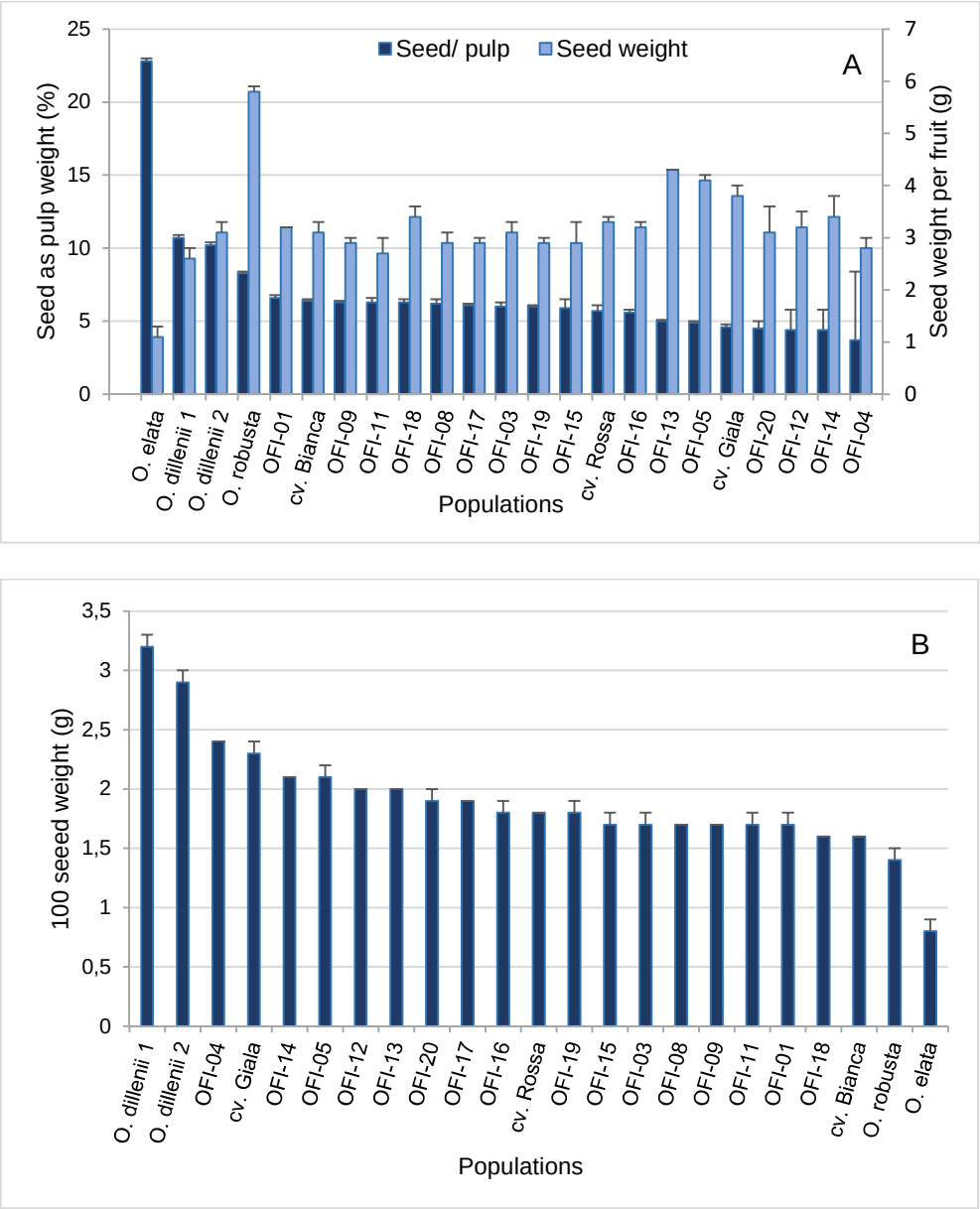

Figure 5.3 A - Seed as pulp weight percentage and seed weight per fruit (g); B – The 100 seed weight (g). Values are the means from the *Opuntia* spp. populations studied (n=30 fruits per population).

The presence of seeds is a deterrent factor to those who consume the fruits of the cactus pear for the first time (Felker *et al.*, 2002). All of the ecotypes in our study had hard seeds, but the percentage of seeds as pulp weight was variable in the OFI ecotypes. The fruit fresh weight (128 g), pulp yield (62.9%) and seed percentage (4.6%) values found in cv. “Gialla” were comparable to the measurements reported in studies made from Argentina (Felker *et al.*, 2002) and in Sicily (Barbera *et al.*, 1992) using the same cv.

Fruit pulp percentages of 50–60% and a minimum of 120 g of fruit fresh weight are required for the export market (Inglese and Gugliuzza, 2002). This minimum weight value was achieved only in a few ecotypes, which may be partially explained by both the low soil water retention capacity and the age of the plants (3 years old).

Total soluble solids, pH, acidity and colour

The cactus pear TSS content varied from 10.6 (*O. dillenii*, the mean value of the two populations was considered) to 15.6% (OFI-03) (Table 5.2). In the OFI, the values ranged from 12.2 (cv. "Rossa") to 15.6% (OFI-03) and statistically significant differences were found among ecotypes, F (18, 48) = 39.6, $p < 0.05$.

Table 5.2 Colorimetric coordinates (L^*, a^*, and b^*), pH, acidity (% citric acid) and total soluble solids (TSS %) from the juice of the different cactus pear populations (n=30, each sample analysed in triplicate).

Population	L^*	a^*	b^*	pH	Acidity (% citric ac.)	TSS (%)
OFI-01	27.44^{abc}	-1.07^{h}	8.09^{c}	6.30^{bcd}	0.05^{d}	14.25^{bcdef}
OFI-03	27.14^{abc}	-0.84^{h}	7.29^{cde}	6.30^{bcd}	0.05^{d}	15.63^{gh}
OFI-04	26.16^{bc}	-1.28^{h}	9.24^{c}	6.10^{fg}	0.05^{d}	15.10^{a}
OFI-05	23.02^{de}	5.18^{ef}	18.55^{a}	6.10^{fg}	0.06^{d}	15.12^{a}
OFI-08	27.73^{abc}	-1.03^{h}	8.19^{c}	6.20^{def}	0.07^{d}	13.70^{defg}
OFI-09	28.41^{a}	-1.07^{h}	6.87^{cde}	6.27^{cde}	0.06^{d}	14.10^{cdef}
OFI-11	27.17^{abc}	-0.80^{h}	5.86^{cdef}	6.20^{def}	0.06^{d}	13.55^{efg}
OFI-12	23.10^{d}	5.47^{de}	19.45^{a}	6.17^{ef}	0.05^{d}	15.07^{ab}
OFI-13	21.84^{de}	8.03^{bc}	16.57^{ab}	6.03^{g}	0.06^{d}	15.05^{ab}
OFI-14	21.00^{e}	7.49^{c}	14.81^{b}	6.20^{def}	0.06^{d}	14.65^{abc}
OFI-15	27.98^{ab}	-1.12^{h}	8.06^{cd}	6.27^{cde}	0.05^{d}	13.47^{fgh}
OFI-16	26.61^{abc}	-1.13^{h}	8.32^{c}	6.47^{a}	0.05^{d}	15.10^{a}
OFI-17	25.74^{c}	-0.89^{h}	7.32^{cde}	6.33^{bc}	0.05^{d}	14.35^{abcde}
OFI-18	26.40^{abc}	-0.93^{h}	7.48^{cde}	6.20^{def}	0.06^{d}	13.23^{gh}
OFI-19	27.11^{abc}	-0.97^{h}	7.13^{cde}	6.30^{bcd}	0.06^{d}	14.37^{abcde}
OFI-20	22.19	6.79^{cd}	17.36^{ab}	6.17^{ef}	0.06^{d}	13.05^{gh}
OFI, cv. "Bianca"	26.60^{abc}	-1.01^{h}	7.06^{cde}	6.40^{ab}	0.07^{d}	13.72^{defg}
OFI, cv. "Gialla"	22.43^{de}	6.79^{cde}	17.53^{ab}	6.10^{fg}	0.06^{d}	14.67^{abc}
OFI, cv. "Rossa"	14.84^{f}	9.78^{ab}	4.60^{defg}	5.90^{h}	0.06^{d}	12.22^{i}
O. robusta	13.42^{fg}	5.45^{de}	2.75^{fg}	5.83^{h}	0.07^{d}	14.42^{abcd}
O. dillenii, OD-1	12.54^{g}	3.20^{gf}	2.07^{g}	3.27^{j}	0.62^{b}	10.37^{j}
O. dillenii, OD-2	12.62^{g}	2.47^{g}	2.02^{g}	3.22^{j}	0.73^{a}	10.75^{j}
O. elata	14.87^{f}	11.66^{a}	4.57^{efg}	4.20^{i}	0.36^{c}	12.70^{hi}

OFI – *Opuntia ficus-indica*; OD – *Opuntia dillenii*. Means with different alphabetic superscripts in same line differ significantly ($P<0.05$).

In the case of the cv. "Rossa", the lowest value we obtained for the TSS could partially be explained by the fact that the fruits were harvested earlier (at commercial maturity) compared to the other ecotypes of OFI, which were harvested at physiological maturity. Indeed, TSS is a variable parameter that depends on the maturity stage and fruit

metabolism (Albano *et al*., 2015). Apart from that difference, we can assume that the observed variations in the different parameters reflected differences at the genotype level since the studied ecotypes were grown in the same soil and the climate conditions and the fruits were harvested at approximately the same physiological state.

The pH values found in *O. dillenii* agreed with the values reported in previous studies (Medina *et al*., 2007). Considering the OFI populations, the pH varies from 5.90 (cv. "Rossa") to 6.47 (OFI-16) (Table 5.2). In this species, the pH of the fruit usually increases from 5 to a range of approximately 5.5–6.5 during ripening (Albano *et al.*, 2015). In our data, *O. dillenii* and *O. elata* showed the highest acidity values (0.68 and 0.36, respectively), while in OFI ecotypes, the acidity values ranged between 0.05 and 0.07% citric acid (Table 5.2). In the populations we studied, the pulp fruit colour varied from white to purple (Table 5.2). The highest *L** values were observed in the white pulp populations. The populations with purple, red and orange coloured fruits had the highest *a** values, while the populations with white pulp fruits had the lowest ones. The red and purple fruits had the lowest *L** and *b** values.

Ascorbic acid

The highest AA content was observed in *O. elata* followed by *O. dillenii* (Table 5.3). The AA content of OFI ecotypes ranged from 180 (OFI-20) to 344 mg kg^{-1} FW (OFI-16), and significant differences among the populations were found, F (18, 38) = 19.3, $p < 0.05$. The cv. "Bianca" had a higher AA content than the cvs. "Gialla" and "Rossa". The values for the AA content that we found in the *O. dillenii* and the OFI ecotypes (Table 5.3) were higher than the content found in previous studies for both species (Medina *et al*., 2007). A similar value for AA content in the cv. "Gialla" and a higher value for a purple OFI cv. compared to the cv. "Rossa" were reported (Albano *et al*., 2015). The differences found among the OFI populations in AA content could be attributed to differences at the genotype level. In our study, an underestimation of the cv. "Rossa" AA content cannot to be excluded since the fruits were purchased from a local producer and a few days passed between harvest and the juice analysis. Indeed, the AA content has been shown to decline slightly a few days after harvesting in fresh cut summer fruits (Allegra *et al*., 2015).

Total phenolic compounds

O. dillenii had the highest values of TPC followed by *O. elata* (Table 5.3). In the OFI ecotypes, significant differences were found among ecotypes, F (18, 38) = 20.8, $p < 0.05$, and the TPC ranged from 617 (cv. "Gialla") to 981 mg GAE kg^{-1} FW (OFI-19). The TPC in the cv. "Rossa" and in the cv. "Bianca" were 788 and 870 mg GAE kg^{-1} FW, respectively.

The TPC found in the *O. dillenii* and the *O. robusta* ecotypes were higher than the values reported in previous studies for the same species (Medina *et al*., 2007; Serra *et al*., 2013). A moderate variation in TPC was found among the OFI ecotypes that we studied. Nevertheless, the TPC values were similar to the values previously reported by other authors (Stintzing *et al*., 2005; Saénz *et al*., 2009) in OFI fruits. The fruits from cv. "Rossa" were purchased at a local producer, and a decrease in TPC after harvest could explain the low TPC values that were obtained. As stated previously (Allegra *et al*., 2015), polyphenol content significantly decreases after 3 days of storage. In *O. elata*, the highest TPC values that were found may have resulted from whole fruit processing, since in *Opuntia* spp. fruits, the TPC is higher in the peel than in the pulp (Yeddes *et al.*, 2013). Our results showed that the TPC was higher in *O. dillenii* and *O. elata* compared to the OFI ecotypes, and the differences in TPC values found in the OFI populations could be attributed to differences at the genotype level. A positive correlation was found between total phenols and ascorbic acid content (R^2 = 0.81). In general, cultivars that contained the highest vitamin C levels had the highest phenol and β-carotene contents (Yahia and Mondragón-Jacobo, 2011).

Table 5.3 Ascorbic acid (mg kg^{-1} FW), total phenolic compounds (mg GAE kg^{-1} FW), DPPH Antioxidant Scavenging Capacity (IC_{50}, g L^{-1}) and betalains content (mg L^{-1}), from the juice of the different cactus pear populations studied (n=30, each sample analysed in triplicate).

Population	Ascorbic acid (mg kg^{-1} FW)	Total Phenolic compounds (mg GAE kg^{-1} FW)	DPPH ASC IC_{50} (g L^{-1})	Betalains	
				Betaxanthins (mg L^{-1})	Betacyanins (mg L^{-1})
OFI-01	204.2^{gh}	827.0^{def}	0.88^{fghi}	5.83^{e}	6.82^{d}
OFI-03	204.2^{gh}	672.7^{def}	0.88^{fghi}	5.86^{e}	6.86^{d}
OFI-04	242.7^{efgh}	650.0^{def}	1.04^{bcde}	6.00^{e}	6.48^{d}
OFI-05	201.8^{gh}	630.7^{f}	0.97^{defg}	44.72^{de}	9.87^{d}
OFI-08	198.8^{gh}	863.3^{def}	0.81^{i}	5.14^{e}	6.14^{d}
OFI-09	252.7^{efg}	833.8^{def}	1.04^{bcde}	5.58^{e}	6.54^{d}
OFI-11	224.3^{fgh}	822.1^{def}	0.98^{cdef}	5.46^{e}	6.50^{d}
OFI-12	206.3^{gh}	633.9^{ef}	0.65^{j}	50.99^{de}	8.27^{d}
OFI-13	201.6^{gh}	641.9^{def}	0.85^{hi}	46.39^{de}	6.63^{d}
OFI-14	241.8^{efgh}	829.0^{def}	1.09^{abc}	63.25^{d}	12.38^{d}
OFI-15	230.7^{fgh}	890.9^{def}	1.06^{bcd}	6.11^{e}	7.19^{d}
OFI-16	344.1^{d}	886.9^{def}	0.99^{cde}	6.67^{e}	7.82^{d}
OFI-17	247.9^{efg}	795.7^{def}	0.83^{hi}	6.37^{e}	7.35^{d}
OFI-18	278.3^{ef}	850.4^{def}	0.99^{cdef}	6.26^{e}	7.63^{d}
OFI-19	223.8^{fgh}	981.0^{de}	0.93^{efgh}	5.50^{e}	6.47^{d}
OFI-20	180.3^{h}	734.4^{def}	1.12^{ab}	41.25^{de}	5.06^{d}
OFI, cv. "Bianca"	299.9^{de}	870.2^{def}	$0,78^{i}$	5.87^{e}	6.77^{d}
OFI, cv. "Gialla"	219.5^{fgh}	617.0^{f}	0.87^{ghi}	40.97^{de}	9.79^{d}
OFI, cv. "Rossa"	206.9^{gh}	785.6^{def}	0.80^{i}	51.05^{de}	84.17^{d}
O. robusta	222.2^{fgh}	982.8^{d}	1.18^{a}	211.25^{c}	434.81^{c}
O. dillenii, OD-1	541.6^{a}	3790.3^{a}	0.06^{l}	575.93^{b}	1516.98^{b}
O. dillenii, OD-2	456.3^{c}	3403.9^{b}	0.07^{l}	778.56^{a}	1675.36^{a}
O. elata	896.9^{a}	2879.3^{c}	0.47^{k}	35.51^{de}	117.99^{d}

DPPH ASC IC_{50} – DPPH Antioxidant Scavenging Capacity IC_{50}; FW – Fresh weight; GAE - Gallic acid equivalents; OFI – *Opuntia ficus-indica*; OD – *Opuntia dillenii*. Means with different alphabetic superscripts in same line differ significantly (P<0.05)

Betalains

Variations in BC were found among the different populations that were studied. *O. dillenii* ecotypes had the highest values of BC, followed by *O. robusta*, *O. elata*, and the lowest contents were found in the OFI populations with white pulp fruits (Table 5.3). Significant differences for yellow-orange betaxanthins and red-violet betacyanins contents were found among the OFI populations, $F = (18, 38) = 105.74$, $p < 0.05$ and $F = (18, 38) = 173.79$, $p < 0.05$, respectively. In the OFI fruits with orange pulp, a higher content of betaxanthins was found compared to white pulp fruits, and the cv. "Rossa" had a higher betacyanin content compared to other ecotypes. In the three cultivars "Bianca", "Gialla" and "Rossa", the betaxanthin contents were 5.9, 40.9 and 51.1 mg L^{-1}, and the betacyanin contents were 6.8, 9.8, and 84.2 mg L^{-1}, respectively. In the group of orange pulp fruits, the ecotype OFI-14 had the highest BC content followed by the ecotype OFI-12. The sequence of BC content in decreasing order was *O. dillenii*, *O. robusta*, *O. elata*, cv. "Rossa", orange pulp populations of OFI and, finally, white pulp populations of OFI. This ranking was similar to one published by Stintzing *et al*. (2005). The BC of *O. robusta* and orange pulp populations (mean value of 56.6 mg L^{-1}), were lower than the values reported in previous studies for similar populations (Stintzing *et al*., 2005). Nevertheless, the BC of cv. "Rossa" was slightly higher than the value reported by the same authors for a red pulp clone. The betalain values found in *O. dillenii* populations were comparable to those reported for *O. stricta* (Castellar *et al*., 2012). The betalain content was affected by factors such as variety (Stintzing *et al*., 2005), stage of maturity (Castellar *et al*., 2012), and climate or geographic site of production (Sumaya-Martínez *et al*., 2011).

DPPH radical scavenging activity

The antioxidant activity of *Opuntia* spp. juice extracts is shown in Table 5.3. The purple juice extract from *O. dillenii* had the highest antioxidant activity (lower value of IC_{50}) followed by the extract from *O. elata*. In the OFI ecotypes, significant differences were found among populations, $F = (18, 38) = 30.3$, $p < 0.05$. The lowest value of IC_{50} was found in the OFI-12 ecotype, and the highest value was found in the OFI-20 ecotype. Interestingly, the extract from the red juice of *O. robusta* had higher values of IC_{50} (lower antioxidant activity) compared to juice extracts from the OFI ecotypes.

The antioxidant activity of the fruit extracts was stronger in the purple-skinned fruits (*O. dillenii*) compared to other populations. This result was consistent with the total phenol and betalain contents of purple-skinned fruits. Positive correlations between 1-IC_{50} and the TPC ($R^2 = 0.87$), betacyanin ($R^2 = 0.81$), betaxanthin ($R^2 = 0.78$) and ascorbic acid ($R^2 = 0.64$)

were found. Negative correlations between 1-IC_{50} and both the L^* (R^2 = -0.60) and a^* (R^2 = -0.54) colorimetric coordinates were found.

Multivariate analysis

The PCA correlation matrix showed that all variables had at least one correlation coefficient greater than 0.3. The overall Kaiser-Meyer-Olkin (KMO) measure was 0.83 with individual KMO measures all greater than 0.7 and classifications of 'middling' to 'meritorious' according to the Kaiser method (1974). Bartlett's test of sphericity was statistically significant ($p < 0.05$), which indicated that the data were likely factorable. The PCA revealed two components with eigenvalues greater than one, which explained 85.2% of the total variance. The screen plot indicated that two components should be retained, and Varimax orthogonal rotation was employed to help interpret these results. The first component explaining 67.8% of variation was represented by the betaxanthin and betacyanin contents, acidity, pH, TPC, DPPH ASC (IC_{50}), TSS, and fruit length. The second component (with 17.4% of the total variation) was explained by the diameter, weight of the fruit, pulp weight, seed weight, amount of seeds as pulp percentage and AA content.

The hierarchical cluster analysis highlighted an overall pattern of genetic diversity and the relationship between germplasm accessions. The clustering analysis (Fig. 5.4) revealed that the 23 genotypes could be classified into four major groups. Cluster 1 comprised all OFI genotypes, cluster 2 comprised *O. robusta*, cluster 3 included the two populations from the species *O. dillenii* and finally the *O. elata* population was included in cluster 4. Four subgroups were found in cluster 1, which were the white pulp fruits (including cv. “Bianca”), the orange pulp fruits (including cv. “Gialla”), the cv. “Rossa”, and the ecotype OFI-04. The latter ecotype was distinguishable from the others due to the quantitative and qualitative characteristics of its fruit, and it could be considered a new variety. The distribution of genotypes in the dendrogram indicates that geographical origin was unrelated to the clustering pattern.

The Mediterranean region is prone to global climate change, which is expected to deeply affect the area in the near future. OFI and related species, which are characterized by their minimal water requirements, rustic durability and adaptability to high temperatures, could play an important role in the land use in the marginal areas of this region. A considerable genetic variation in the concentration of bioactive compounds and morphological characteristics of the fruits was observed among different *Opuntia* spp. and among different OFI ecotypes. *Opuntia*. spp. are an interesting source of phenolic compounds, betalains, and ascorbic acid. Additionally, moderate consumption of cactus pear fresh fruit can provide important antioxidant intake. The Iberian Peninsula is likely a source of additional

morphological and genetic variability inside this genus, and further germplasm collection as well as harvest and subsequent characterization of ecotypes (particularly OFI) should be undertaken to better understand the *Opuntia* spp. in this area.

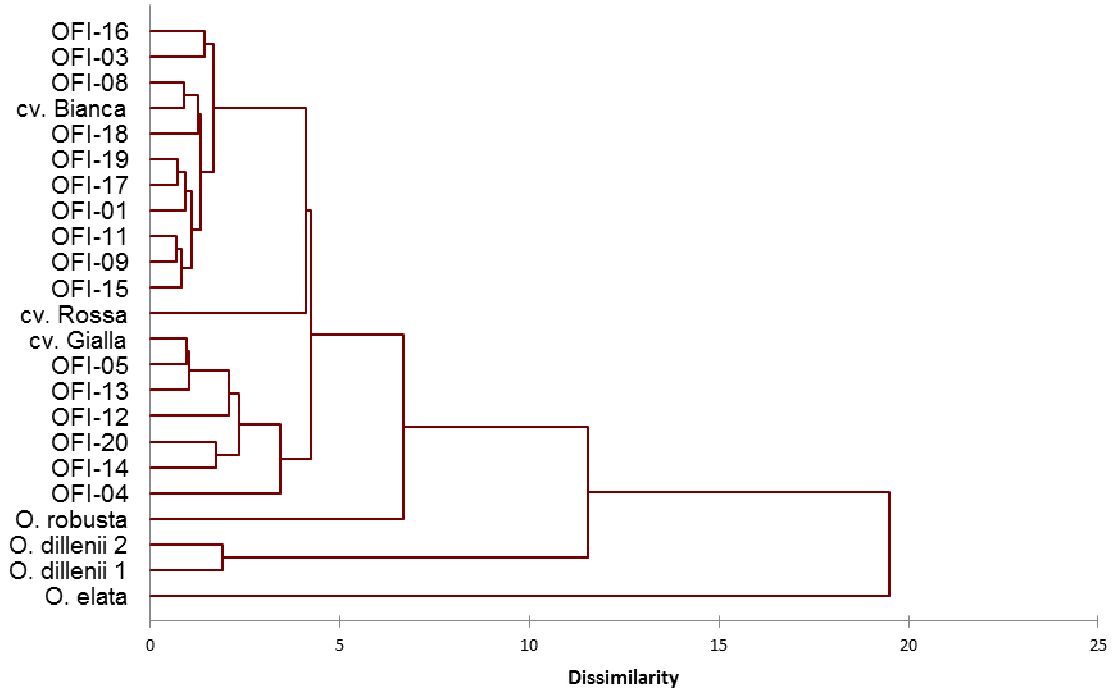

Figure 5.4 Hierarchical clustering analysis for 20 *Opuntia* spp. Portuguese ecotypes and three *O. ficus-indica* cultivars ("Bianca", "Gialla" and "Rossa") based on fruit morphological and chemical characteristics pairwise Euclidian distances.

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6. *Opuntia* spp. Portuguese ecotypes show low levels of genetic diversity with SSR markers

This chapter is based on the following manuscripts:

Reis, C.M.G., Raimundo, J. & Ribeiro, M.M. (2018). Assessment of genetic diversity in *Opuntia* spp. Portuguese populations using SSR molecular markers. *Agronomy*, 8, 55-65; https://doi.org/10.3390/agronomy8040055.

Raimundo, J., **Reis, C.M.G**, & Ribeiro, M.M. Rapid, simple and universal method for DNA extraction from *Opuntia* spp. fresh cladode tissues suitable for PCR amplification. *Submitted.*

6.1 Summary

The *Opuntia* spp. were introduced to the Iberian Peninsula most likely in the beginning of the 16th century, after the discovery of America, spreading afterwards throughout the Mediterranean basin. We analysed for the first time the genetic diversity in a set of 19 Portuguese *Opuntia* spp. ecotypes from the species *O. ficus-indica*, *O. elata*, *O. dillenii* and *O. robusta* using nuclear microsatellite (nuSSR) markers. The Italian cultivars “Bianca”, “Gialla” and “Rossa” were included in the study for comparison purposes. The SSR amplifications produced from five to 16 alleles, with an average of 9.2 alleles per primer pair. The hierarchical clustering analysis revealed four major groups that clearly separated the four *Opuntia* species. Among the *O. ficus-indica* ecotypes, two sub-clusters were found, one including the white pulp fruits (with cv. “Bianca”) and the other with the orange pulp ones and including the cv. “Gialla”, the cv. “Rossa”, and one pale yellow pulp ecotype. No genetic differences were found between the inermis form, *O. ficus-indica* f. *ficus-indica*, and the rewilded spiny one, *O*. *ficus-indica* f. *amyclaea*. The dendrogram indicated that the clustering pattern was unrelated to geographical origin. The results revealed a low level of genetic diversity among the Portuguese ecotypes of *O. ficus-indica*.

6.2 Introduction

The genus *Opuntia* is in the family Cactaceae (subfamily Opuntioideae, tribe Opuntieae). The most widespread and economically important species is *Opuntia ficus-indica* (L.) Miller (OFI), with the domestication centre localized in central Mexico (Griffith, 2004). Platyopuntias are the most widespread cacti out of their original range, with the dissemination by humans initiated after the conquest of the New World by Europeans (Casas and Barbera, 2002). In milder Mediterranean areas, the plants have found optimal environmental conditions, spreading and naturalizing, becoming typical in the Mediterranean landscape (Barbera *et al.*, 1992).

Portugal has the following *Opuntia* species: *O. ficus-indica* (L.) Miller, *O. dillenii* (Ker-Gawler) Haw., *O*. *robusta* Wendl. ex Pfeiffer and *O. elata* Link & Otto ex Salm-Dick. *Opuntia ficus-indica* has two forms, the inermis, typical form, *O. ficus-indica* f. *ficus-indica* (L., Mill., 1768), and the rewilded spiny one, *O*. *ficus-indica* f. *amyclaea* (Ten. Schell, 1907) (Kiesling, 1998).

Plant genetic resources play an important role in the improvement of wild and cultivated plants. Among the molecular markers, microsatellites are widely used for individual genotyping, germplasm evaluation, genetic diversity studies, genome mapping, and phylogenetic and evolutionary studies (Kalia *et al.*, 2011).

The Cactaceae present some peculiarities regarding molecular marker studies. First, the isolation of DNA from cacti is notoriously difficult because of high amounts of polysaccharides and secondary metabolites (De la Cruz *et al.*, 1997; Mondragón-Jacobo *et al.*, 2000). Additionally, these contaminants inhibit the action of restriction enzymes and Taq polymerases (Pandey *et al.*, 1996). Second, polyploidy is a common phenomenon throughout the tribe Opuntieae, which presents some drawbacks in the analysis of codominant markers, such as microsatellites (SSR). Diploids ($2n = 2x = 22$) are relatively rare in this tribe composing only 26.2% of the 164 species with reported chromosome counts (Majure *et al.*, 2012). Polyploid taxa within *Opuntia* spp. range from triploid ($2n = 3x = 33$) to octoploid ($2n = 8x = 88$), and many species have multiple ploidy levels (Pinkaya, 2002; Majure, et al, 2012).

Until now, few studies have been conducted to identify genomic microsatellites and to develop markers in *Opuntia* spp. Helsen *et al.* (2007) developed 16 SSR markers from *O. echio*s, and Erre *et al.* (2011) obtained ten SSR markers from *O. ficus-indica*. Helsen *et al.* (2009) used the SSRs markers previously obtained to distinguish between two morphologically distinct *O. echios* botanical varieties (*echios* and *gigantea*) native to the Galapagos Islands. With the same set of primers, Caruso *et al.* (2010) investigated the level

of intraspecific genetic diversity among *O. ficus-indica* cultivated varieties and some related species from the Mediterranean region and Mexico. They stated that the genotypes cultivated in Mexico showed high levels of diversity, whereas most of the spineless accessions collected in other countries had a very narrow genetic base. Samah *et al.* (2016) used 13 SSR molecular markers previously developed (Helsen *et al.*, 2007; Erre *et al.*, 2011) to study the genetic diversity of Mexican *Opuntia* germplasms with agronomic and economic importance.

To our knowledge, no molecular-based studies have been conducted with Portuguese ecotypes of *Opuntia* spp., and therefore, the genetic relationships and population structure are unknown. Therefore, we were motivated by the lack of genetic studies on Portuguese *Opuntia* spp. ecotypes to investigate this genus aggregate at the population level using SSR markers.

The objectives of this study were to (i) evaluate the genetic relationships among ecotypes of different *Opuntia* species, (ii) assess the molecular diversity of Portuguese *O. ficus-indica* ecotypes, and (iii) compare their relationships with three improved *O. ficus-indica* Italian cultivars.

6.3 Materials and methods

Plant material and DNA extraction

The accessions studied were from four *Opuntia* species, *O. ficus-indica, O. elata, O. dillenii,* and *O. robusta*, collected from several places throughout Portugal and established in a provenance trial at the Escola Superior Agrária de Castelo Branco (ESACB) (Table 6.1). A total of 22 populations each containing 15 individuals were analysed. The Italian cultivars "Bianca", "Gialla" and "Rossa" were used for comparison purposes.

DNA was extracted from 100 mg of freshly sampled chlorenchyma tissue and was extracted from all fifteen individuals in each population. DNA was extracted with a DNeasy® Plant Mini Kit with slight modifications, primarily in the incubation time and in the centrifugation speed, duration, and intensity (our unpublished study).

For samples with high content of mucilage (samples from *O. elata* and *O. dillenii*), a lysis process with CTAB and SDS and the combination of this improved lysis with a DNeasy® Plant Mini Kit were used. The lysis buffer supplied with the DNeasy® Plant Mini Kit was replaced with 700 µL of CTAB lysis buffer, 50 µL of 0.2 M SDS and 4 µL of RNase A. A step to remove proteins was performed by adding chloroform:isoamyl alcohol (24:1), with an extra centrifugation at 10,000 rpm for 10 min, conducted after the kit buffer was used for this purpose (Buffer AP2). Afterwards, the lysate was pipetted into a QIAshredder spin

column placed in a 2 mL collection tube, and the remaining steps of the standard protocol of the kit were followed.

The extraction products were dissolved in 75 µL of TE buffer to maximize overall DNA yield. DNA yield was determined by spectrophotometry (mySPEC VWR®, Leuven, Belgium), whereas DNA purity was estimated according the A260/A280 ratio. DNA was also quantified by visual comparison with lambda DNA samples on GreenSafe premium (Nzytech, Lisbon, Portugal) stained agarose 0.8% gels, and documented with the Micro Doc gel documentation system (Cleaver Scientific, Warwickshire, United Kingdom).

Table 6.1 Genotypes, pulp colour and geographic origin of the studied *Opuntia* spp. populations.

Genotype code	Pulp colour	Origin	Altitude (m)	Geographic coordinates	
				Latitude	Longitude
OFI-01ª	White	Alcochete	25	38°43'32.14"N	8°57'58.22"W
OFI-03	White	Cascais	185	38°45'23.18"N	9°27'38.48"W
OFI-04	Pale yellow	Portalegre	372	39°16'22.45"N	7°26'13.12"W
OFI-05	Orange	Arronches	293	39° 5'21.06"N	7°12'7.05"W
OFI-08	White	Melides	29	38° 8'28.91"N	8°44'14.28"W
OFI-09	White	Santo André	25	38° 4'38.13"N	8°46'38.08"W
OFI-11	White	Albufeira	61	37° 5'23.33"N	8°17'27.03"W
OFI-12	Orange	Cacela-a-Velha	20	37° 9'22.50"N	7°32'47.98"W
OFI-13	Orange	Monforte da Beira	260	39°45'8.34"N	7°16'54.83"W
OFI-14	Orange	Idanha-a-Velha	275	39°59'57.30"N	7° 9'3.51"W
OFI-15	White	Ponte de Sor	125	39°16'15.45"N	8° 0'44.72"W
OFI-16	White	Coruche	76	38°54'40.93"N	8°37'17.00"W
OFI-17	White	Castelo Branco	402	39°48'58.84"N	7°29'37.85"W
OFI-18	White	Reg. Monsaraz	223	38°27'27.04"N	7°39'21.77"W
OFI-19	White	Alvega	105	39°27'15.96"N	8° 3'51.88"W
OFI-20	Orange	Madeira	116	32°38'54.18"N	16°57'46.38"W
OFI, cv. “Bianca”	White	Italy	--	--	--
OFI, cv. “Gialla”	Orange	Italy	--	--	--
OFI, cv. “Rossa”	Red	Italy	--	--	--
O. robusta	Red	Castelo Branco	365	39°49'17.00”N	7°27'41.00”W
O. dillenii	Purple	Lagos	48	37° 8’42.24”N	8°40’33.42”W
O. elata	Purple	S. J. Pesqueira	450	41° 9’5.83”N	7°22’5.43”W

OFI – *Opuntia ficus-indica*; OFI-01ª – *Opuntia ficus-indica* f. *amyclaea*; Ell – elliptic; Obl – oblong; Ovd – ovoid; Rou – round.

Microsatellite genotyping

The genomic DNA was amplified using fifteen nuclear microsatellite (nuSSR) markers selected from two primer sets, the OPUNTIA (Helsen *et al.*, 2007) and the OPUFIC (Erre *et al.*, 2011) developed for the species *O. echios* and *O. ficus-indica*, respectively, as previously referred (Table 6.2). To search for polymorphisms and understand the allelic patterns at each locus, the nuSSRs were tested in ten populations collected from the four-studied species.

The amplifications with the OPUNTIA set of primers were conducted in 10 µL of total reaction volume containing 5-15 ng of genomic DNA, 1 U Supreme NZYTaq 2x Colourless

Master Mix® separate $MgCl_2$ (Nzytech, Lisbon, Portugal), 2 mM $MgCl_2$, and 0.2 µM of each primer (Table 6.2). The amplifications were performed on a UNO^{96} Gradient thermocycler (VWR®, Leuven; Belgium) programmed with an initial denaturation step of 10 min at 95 ºC, followed by 35 amplification cycles composed of denaturation (1 min at 95 ºC), annealing (1 min at optimal annealing temperature for each pair, see Table 2) and polymerizing (1 min at 72 ºC). After the amplification cycles, a final extension step was for 10 min at 72 ºC.

Table 6.2 Marker code, primer sequences, repeat unit, size, and annealing temperature of the six OPUNTIA and the nine OPUFIC primers used in this study.

Marker	Primer sequences 5´- 3´	Repeat unit	Size range (bp)	Annealing temp. (ºC)
OA3[a]	F: GTG AGT GCC CAG ATG AAA CT R: TCC TCA ACT TTA TTG TAG CAA GAG	(AG)19	232-312	56
OA5[a]	F: TAT GCA CAA AGC ACC ATG C R: CCA ACC ATA CCA ACT GTA CTG AC	(TA)5	198-380	57
OA9[a]	F: CTA GGC TTC ATC CCA CAT TAG G R: TCC AAA TTC ACC TCC TCT GC	(AG)15	144-174	56
OA11[a]	F: CCT ACA CCT GCT GCC AAT C R: CGA GAC AAA CAT CAG AGG AG	(CT)13TT(CT)2	102-111	56
OA12[a]	F: TAA TCT TAT TCT CAG GTC AGT TAC R: GGT ATC TTG TTA TTC GTT CG	(TC)4C(TC)12	224-272	56
OA13[a]	F: CCA AAT ACC CAG CCC ATA C R: CGA GAA CCT AAC TTC CGA TG	(AG)12	244-274	57
OC1[b]	F: TGG GTG AGA CAA TAT AGT AGA CCA AG R: CTG CCG TGA AAT CTG AAT GG	(CT)16	147-178	52
OC3[b]	F: GCT TTG AAA TGT CTT GTG TGA ATG R: AGT CCT GGG AAT CCT CAA CC	(TG)12	134-162	52
OC4[b]	F: TGC AGT CAG GTT TCT CAT TGT C R: GCC CAA CTC TTA CCC TCT CC	(TG)12	196-218	52
OC9[b]	F: GGC AAT ACC CTG AGT TGA GC R: CCT GAG ACT ACA GCG TGA GGA	(AAG)16	182-236	57
OC10[b]	F: GCT TCC TTC AAT AGC ATG ACC R: TGA GGC TTT ACA TGG CAC AC	(GT)12	213-250	55
OC13[b]	F: GGG CTT TCA ACG ATG CTG R: AAG ACA TAG GTT GGA GAC TCA ATT C	(TC)12(AC)11	138-190	55
OC14[b]	F: AAT TGA CCT CTT CAC GTT ATG C R: GAG AAA GTG AGG CAG ACA ACG	(CTT)7CTT)10	162-262	58
OC15[b]	F: TTA AAC CTG CAC ACC ATT CG R: GTG TGA GGC GAG GTT GCT C	(CT)22	182-213	56
OC17[b]	F: ATG GAT CGT CTT CGT CCT TG R: GAT GTC ACC CCA TTC CAT TC	(AG)13	158-181	55

[a]OPUNTIA set of primers according Helsen *et al.* (2007); [b]OPUFIC set of primers according Erre *et al.* (2011); F – primer forward; R – primer reverse.

The OPUFIC amplifications were conducted in a total reaction volume of 10 µL containing 5-15 ng of genomic DNA, 0.8 U Supreme NZYTaq 2x Colourless Master Mix® separate $MgCl_2$ (Nzytech, Lisbon, Portugal), 2 mM $MgCl_2$, and 0.2 µM of each primer. The amplifications were performed in the same thermocycler programmed with an initial denaturation step for 10 min at 95 ºC, followed by 35 amplification cycles composed of denaturation (30 s at 95 ºC), annealing (30 s at optimal annealing temperature for each pair, see Table 6.2) and polymerizing (30 s at 72 ºC). After the amplification cycles, a final extension step was for 10 min at 72 ºC.

The forward primers were 6-FAM fluorescently labelled, and amplifications were conducted separately for each primer pair. An aliquot of 1.0 µL of PCR product of each primer pair was mixed with 10 µL of formamide and 0.2 µL of ROX-500 size standard. Genotyping was performed with an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and the fragment analysis was performed using GENE-MARKER 1.5 software (SoftGenetics, LLC, State College, PA) and the data manually scored.

Data analysis

Despite the microsatellite codominance, we followed the approach used in polyploids (Caruso *et al.*, 2010; López-Vinyallonga *et al.*, 2015; Pfeiffer *et al.*, 2011; Samah *et al.*, 2016), and the SSR peaks were scored as dominant *loci* to circumvent the polyploidy and to obtain the data for conventional population genetics analysis software. Hence, the pattern of SSR peaks observed at each *locus* was recorded as a qualitative character for the presence (1) or absence (0) and a binary matrix was created. Although scored as dominant markers, microsatellites were more informative than random markers, such as RAPD, because they are more reliable and more precise. Additionally, microsatellite fingerprinting of polyploid plants is a cost efficient and reliable alternative to dominant markers, such as AFLPs, and fewer loci are required than for diploids (Pfeiffer *et al.*, 2011), provided that the microsatellite primers have been developed, which was the case for this study.

The number of alleles and number of rare alleles (alleles with a frequency less than 5%) were calculated for each locus. The primer discriminatory power was evaluated by the polymorphic information content (PIC) and marker index (MI). The PIC was calculated according to Botstein *et al.* (1980):

$$1 - (\sum_{i=1}^{n} P_i^2) - \sum_{i=1}^{n-1} \sum_{J=i+1}^{n} 2\ P_i^2\ P_j^2$$

where P_i and P_j are the population frequency of the i^{th} and j^{th} allele, respectively. The marker index (MI) was calculated as $MI = EMR \times PIC$, and the effective multiplex ratio (EMR) was calculated as follows (Powell *et al.*, 1996; Nagaraju *et al.*, 2001):

$$EMR = n_p \left(\frac{n_p}{n}\right)$$

where n_p is the number of polymorphic loci, and n is the total loci number.

A matrix to evaluate pairwise genetic similarity between accessions was calculated based on the Dice similarity coefficient, which is a band-sharing-based method, using the SIMQUAL NTSYS-pc Version 2.21q subprogram (Rohlf, 2002). The cluster analysis was performed using the unweighted pair group arithmetic mean method (UPGMA) in the SAHN NTSYSpc subprogram. The UPGMA tree topology was verified by comparing the original genetic distance matrix with the cophenetic matrixes obtained from the corresponding dendrograms using the Mantel matrix-correspondence test (Mantel, 1967) and bootstrap analysis with 1,000 permutations.

The Nei's genetic distance matrix obtained with the AFLP-SURV 1.0 software (Vekemans *et al.*, 2002) was used to perform a Principal Coordinate Analysis (PCoA) using GenAlEx 6.501 software (Peakall and Smouse, 2012), and the first two principal coordinates were plotted to indicate the multilateral genetic relationships between them.

The grouping structure was further explored using a locus-by-locus Analysis of Molecular Variance (AMOVA) (Excoffier *et al.*, 1992), conducted with Arlequin 3.5 software (Excoffier and Lischer, 2010). The groups were defined based on the PCoA clustering. Variance components and Φ statistics were estimated for each locus and then combined to produce a synthetic estimator of the among groups differentiation, the Φ_{ct} statistic. The significance values were computed by a permutation test from 1,000 permuted matrices.

6.4 Results

Microsatellite genotyping

Among the 15 SSR primer pairs initially tested on 10 populations, the 9 OPUFIC primer pairs were discarded, because the peak profiles produced were difficult to interpret or were redundant comparatively with those of the OPUNTIA primers and did not provide additional information. The six OPUNTIA primers gave reliable, readable, and reproducible profiles and therefore were used to study the genetic variability and to realize the DNA fingerprint of the 22 *Opuntia* spp. populations (Fig. 6.1). In each studied population, the 15 individuals genotyped had the same SSR profile for each of the six pairs of primers.

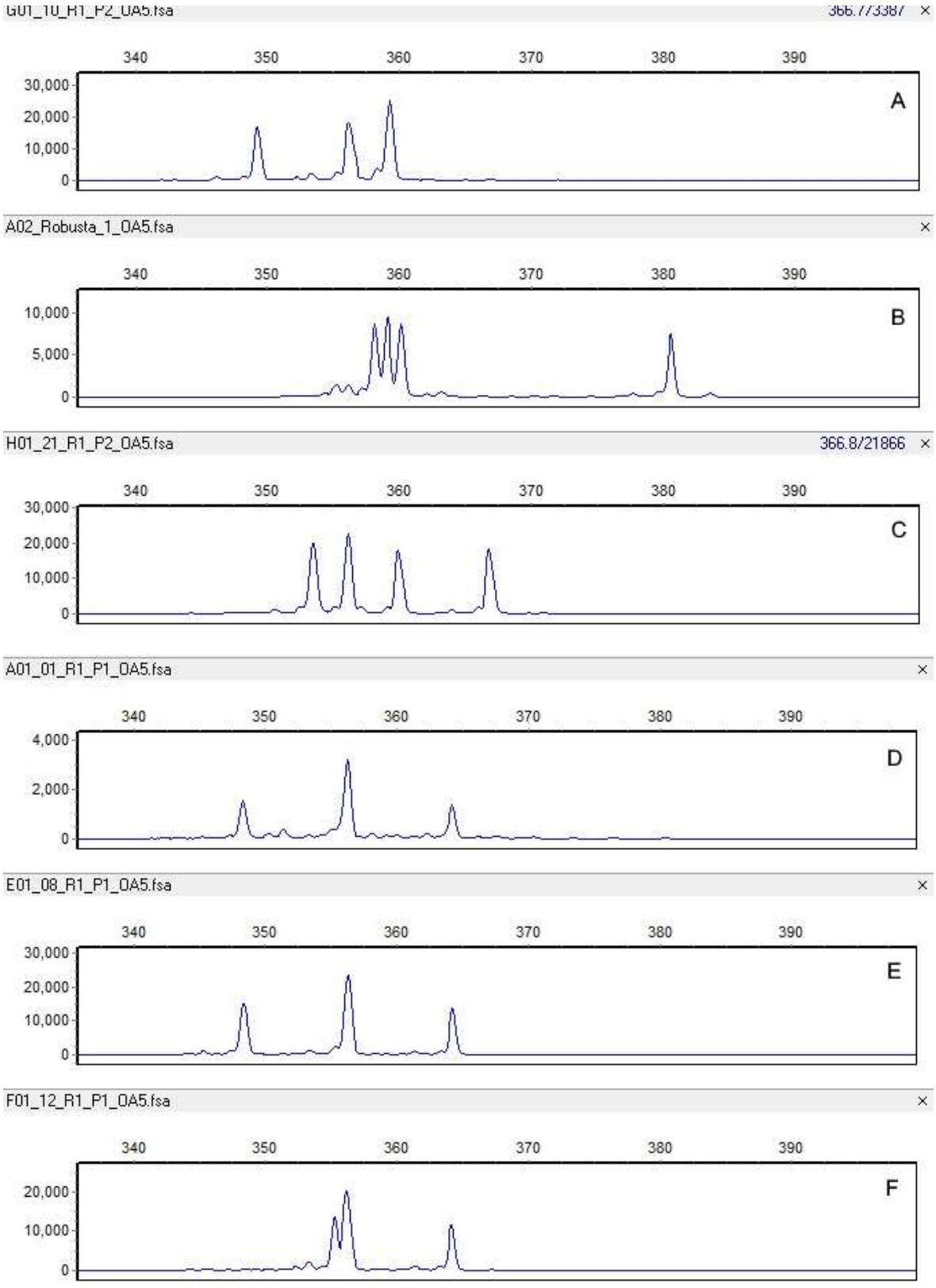

Figure 6.1 SSR electropherograms of OA5 amplicons obtained with DNA extracted from four *Opuntia* species. A – *O. dillenii;* B – *O. robusta*; C – *O. elata*; D – *O. ficus-indica* f. *amyclaea* (OFI-01); E – *O. ficus-indica* f. *ficus-indica* (OFI -08, white pulp); F - *O. ficus-indica* f. *ficus-indica* (OFI-12, orange pulp).

A total of 55 alleles were detected among the 22 populations studied, ranging from 6 (OA3) to 16 (OA12), with a mean of 9.2 alleles per marker (Table 6.3). Twenty-four were rare alleles, representing 43.6% of the total full set of alleles.

The average PIC values ranged from 0.495 (OA9) to 0.882 (OA12), with an average over loci of 0.711 (Table 6.3). The MI values ranged from 3.524 (OA11) to 14.106 (OA12), averaging 6.735 (Table 6.3). All the studied loci were informative because they showed an MI value greater than 3; however, only the OA12 loci generated more than 12 alleles per locus.

Table 6.3 Number of alleles (Na), alleles per individual, number of rare alleles, polymorphic information content (PIC), and marker index (MI) values for the six SSR primer pairs studied.

Marker	Na	Alleles per individual	No. of rare alleles	PIC (average)	MI
OA3	6	2 - 3	3	0.635	3.808
OA5	8	2 - 4	4	0.738	5.903
OA9	10	2 - 5	5	0.495	4.948
OA11	5	2 - 5	1	0.705	3.524
OA12	16	3 - 7	7	0.882	14.106
OA13	10	2 - 5	4	0.812	8.120

Number of rare alleles refers to alleles with a frequency lower than 5%.

Genetic distances and clustering

A genetic similarity matrix based on the Dice coefficient was obtained with the microsatellite data (Appendix 6, Table S6.1). This matrix was used to group all accessions using UPGMA. The tree topology tested with a Mantel test showed a very high correlation ($r = 0.995$; $P < 0.001$). The estimated Dice coefficient among populations varied from 0.255 (the most distant accessions were *O. elata* and the set of white pulp OFI populations) to 1.0 (the various white pulp OFI populations were similar to one another, as were the orange pulp OFI populations), indicating high interspecific genetic diversity but low genetic diversity at the intraspecific level (Appendix 6, Table S6.1). These relationships were supported by the cluster analysis (Figure 6.2).

The hierarchical clustering analysis revealed four major clusters, and the four *Opuntia* species were clearly separated from one another (Figure 6.2). Three branches included the accessions representing each of the species *O. dillenii*, *O. elata* and *O. robusta*. The fourth and larger group included the *O. ficus-indica* cultivars and ecotypes. Among the *O. ficus-indica* ecotypes only two sub-clusters were found: one contained the white pulp fruits (including cv. “Bianca”) and the ecotype OFI-01 (which corresponded to the spinescent form *amyclaea*), and the other contained the orange pulp fruits (including cv. “Gialla”), the cv. “Rossa”, and one ecotype with pale yellow pulp (OFI-04) (Figure 6.2). The distribution of

genotypes in the dendrogram indicated that the clustering pattern was unrelated to geographical origin.

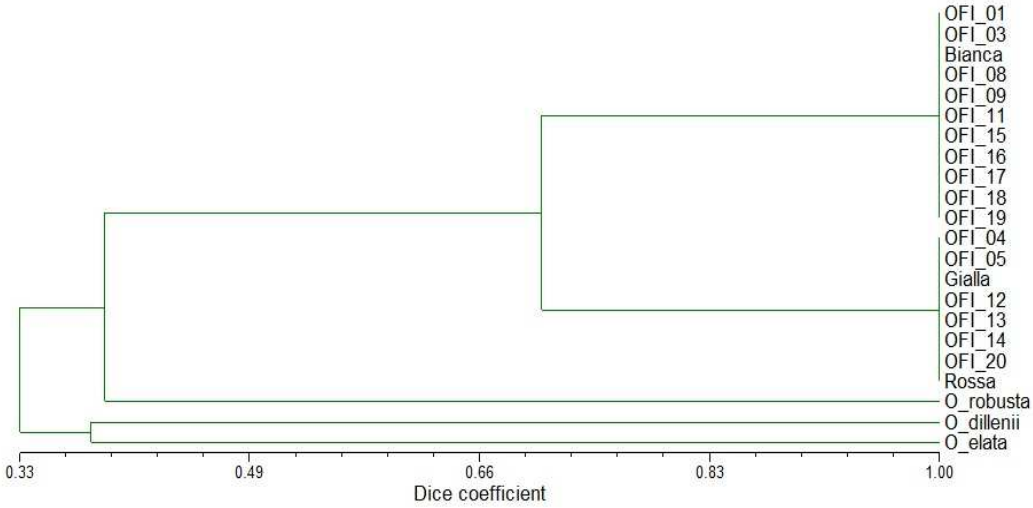

Figure 6.2 Dendrogram of the 22 *Opuntia* spp. populations obtained from SSR markers based on Dice coefficient and using UPGMA as clustering method.

In the two-dimensional PCoA plot, the *Opuntia* spp. populations were divided into five groups, similar to the UPGMA dendrogram pattern (Figure 6.3). The first and second principal axes explained 74.66% and 15.43% of the total molecular variation observed, respectively. The species *O. elata* and *O. robusta* were separated from the other species on axis 1, and *O. dillenii* was separated from the other species on axis 2 (Figure 6.3). The *O. ficus-indica* populations were distributed into two groups, which corresponded to the two sub-clusters obtained in the hierarchical clustering analysis

With the AMOVA, we estimated the variance components and genetic variation among accessions within groups and among groups. In this test, three groups were defined based on the PCoA. The first (A) and second group (B) included the *O. ficus-indica* populations, and the third group (C) contained the three unique populations of the species *O. dillenii*, *O. elata*, and *O. robusta* (Figure 6.3). The AMOVA confirmed a significantly ($P < 0.0001$) high differentiation among groups ($\Phi_{ct} = 0.87$; Table 6.4). However, the variation was low among populations within groups, and no variation occurred within populations

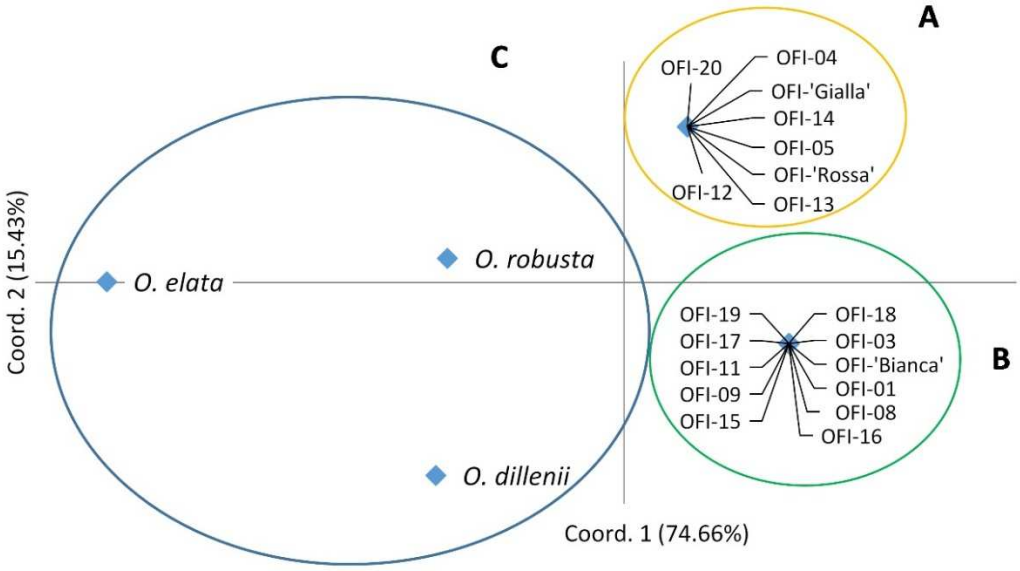

Figure 6.3 Principal coordinates analysis (PCoA) based on Nei's genetic distances between the 22 *Opuntia* spp. populations. OFI – *Opuntia ficus-indica*.

Table 6.4 Analysis of molecular variance (AMOVA) of the *Opuntia* spp. accessions, considering the whole data set clustered in three groups (A, B and C) according to the PCoA analysis. SS = sum of squared deviation, df = degrees of freedom and P = level of probability of obtaining a more extreme component estimate by chance alone.

Source of variation	df	SS	Variance components	% of total variance	P
Among groups (Φ_{ct} = 0.87)	2	1552.696	7.85732	86.72	<0.0001
Among populations within groups	19	339.512	1.20363	13.28	<0.0001
Within populations	304	0,000	0.00000	0	<0.0001
Total	325	1892.208	9.06095		

6.5 Discussion

The genetic diversity of 19 Portuguese *Opuntia* spp. ecotypes and three Italian cultivars was assessed using the six SSR markers designed from the Galapagos *O. echios* by Helsen *et al.* (2007) and tested by Caruso *et al.* (2010) in another *Opuntia* species. A unique microsatellite pattern was found for the different species studied, clearly differentiating at the species level. Among the *O. ficus-indica* ecotypes, two sub-clusters were identified, one including the white pulp fruits (with cv. "Bianca") and the other with the orange pulp fruits, including the cv. "Gialla", the cv. "Rossa", and one pale yellow pulp ecotype. However, at the intrapopulation level, the microsatellite pattern over loci was the same, and the

individuals could not be distinguished, suggesting that they could be recent vegetative clones. The results revealed a low level of genetic diversity among the Portuguese ecotypes of *O. ficus-indica* and the Italian cultivars.

The spinescent *O. ficus-indica* f. *amyclaea* (OFI-01) had the same microsatellite fingerprint as that of the *O. ficus-indica* f*. ficus-indica* spineless ecotypes, and the clustering did not reflect the spinescence character. Caruso *et al.* (2010) reached similar conclusions in a previous study of the level of intraspecific genetic diversity among *O. ficus-indica* cultivated varieties and some related species. These authors obtained results from a cluster analysis that clearly diverge from the current taxonomy, which classifies several *Opuntia* species based on morphological parameters such as the presence/absence of spines.

We note that the markers used for this study are putatively neutral and they might not necessarily reflect morphological characteristics, which are influenced by the environment and are under selection because they are products of transcription or translation. Therefore, although these markers can provide invaluable insights into parameters such as genetic diversity within populations, genetic differentiation among populations, inbreeding, and demographic events, they provide limited insight into adaptive evolution and evolutionary potential (Kirk and Freeland, 2011).

This study represents the first report on the population structure and genetic diversity of Portuguese *Opuntia* ecotypes accessed by SSR markers. The results obtained with the six SSR markers revealed a narrow genetic base of diversity among the *O. ficus-indica* accessions. Our results are consistent with previous studies (Caruso *et al.*, 2010; Samah *et al.*, 2016); generally, high levels of diversity are found among the Mexican cultivated genotypes, whereas most of the spineless accessions collected in other countries, primarily in the Mediterranean region, have a very narrow genetic base. Similarly, using microsatellites, lower genetic diversity was found in the Portuguese landrace of the species *Eucalyptus globulus* Labill., introduced during the 19th century, than that found in native species (Freeman *et al.*, 2007).

In the Mediterranean Basin, some *Opuntia* spp. (primarily *O. ficus-indica* Mill.) were introduced five centuries ago from their areas of origin (Casas and Barbera, 2002), and the cactus pear was so well-suited to the environmental conditions that they rapidly became naturalized. However, on the Iberian Peninsula, *O. ficus-indica* rarely expands by seed germination because of thermal and hygrometric conditions, which are seldom optimal for reproduction (Blasco *et al.*, 2015; Nieddu and Chessa, 1997). Although the occurrence of apomixis is possible in *O. ficus-indica* (Mondragón-Jacobo, 2001), the absence of intrapopulation variability that we found supports the predominance of asexual propagation and the absence of natural multiplication by seed germination.

Opuntia ficus-indica areas of occurrence are related to human activity and its propagation is maintained by asexual reproduction (Blasco *et al.*, 2015). *Opuntia ficus-indica* is found on roadsides and paths due to typical ruderal behaviour and is cultivated for edible fruit production and hedge establishment. In some areas, the cactus is abundant in places with steep slopes on which the cladodes break off and easily root, forming conspicuous patches (Erre *et al.*, 2009). In the Mediterranean region, naturalized populations of *Opuntia* spp. show a lower genetic heterogeneity than that of the areas of origin. Difficulties linked to the reproductive process, such as cleistogamy and polyembryony, in addition to lack of rain during summer and decreasing temperatures in autumn, have essentially eliminated the production of plants from seed and as a result limited the extent of new genetic variability (Chessa and Nieddu, 2002).

Nevertheless, although the genetic diversity among the studied accessions was low, the Portuguese ecotypes showed some phenotypic variability in plant vigour, cladode shape, presence or absence of spines, spine length, corolla colour, pulp colour and fruit ripening time (Reis *et al.*, 2018). Variation at neutral loci is not influenced by natural selection but primarily by mutation and genetic drift (Kimura, 1983). Moreover, high migration rates may erase population divergence. The balance between mutation and selection and among different selective pressures shapes the variation of adaptive traits. Therefore, selection acts differently along the genome, whereas migration acts evenly. Neutral or nearly neutral molecular markers are unlikely to accurately predict patterns of variation in quantitative traits when selection and drift are the acting forces (Reed and Frankham, 2001).

A good amplification of the microsatellites occurred in the studied species with the same set of primer pairs, which indicated their genetic proximity. Known primers are not likely to amplify the same locus across related taxa unless the flanking regions in which priming sites are located are highly conserved (Ellegren 1992), which typically occurs in closely related species (Kijas *et al.* 1995). Similarly, in the study by Samah *et al.* (2016), the 13 primers developed for *O. echios* (Helsen *et al.*, 2007) and *O. ficus-indica* (Caruso *et al.*, 2010; Erre *et al.*, 2009) successfully amplified fragments of all genotypes, showing a high degree of cross-transferability among the analysed species of the genus *Opuntia*. Moreover, some of the primers used generated amplicons in three cacti included as out-groups: *Cylindropuntia*, *Pitahaya*, and *Pitaya*. Other studies have also demonstrated the transferability of SSR markers among species and genera (Wang *et al.*, 2008; Ekué *et al.,* 2009).

Speciation in the genus *Inga* (Fabaceae) is recent and is considered a classic example of a recent radiation with evidence for many species arising within the last 10 million years, some of them as recently as 2 million years ago (Richardson *et al.,* 2001). A similar rapid

and recent burst of diversification of extant species may have occurred in the genus *Opuntia*, resulting in a poorly resolved phylogeny.

Additionally, the *O. ficus-indica* taxonomic concept may circumscribe a non-monophyletic group of convergent cultivars derived from different parental species selected for spineless cladodes and desirable fruits, artificially converging further upon these traits, and propagated clonally to the present day (Griffiths, 2004).

Recent advances, such as next-generation sequencing, are expected to permit the development of non-neutral markers by targeting genetic regions that are directly influenced by natural selection (Kirk and Freeland, 2011). The large *O. ficus-indica* octoploid genome (4 Gbp) makes comprehensive and accurate de novo genome sequencing difficult. However, the diploid reference species *O. cochenillifera* genome is currently undergoing PacBio SMRT sequencing and most likely will help in the assembly of an existing data set from *O. ficus-indica* (Mayer *et al.*, 2016). The knowledge generated on structural and functional genomics will allow systematic development of gene-targeted and functional markers, which are derived from polymorphic sites within genes (Andersen and Lübberstedt, 2003). Such techniques have the potential to generate phenotypically linked functional markers, particularly when fingerprints are generated from the transcribed or expressed region of the genome (Pozcai *et al.*, 2013).

The molecular characterization of ecotypes could assist plant breeders with a better understanding of the existing genetic variability. The collection and characterization of germplasm from native and naturalized populations, together with continued efforts at *Opuntia* breeding, are required to develop new cultivars both for fodder and fruit production.

In this study, nuSSR markers revealed a low level of intraspecific genetic diversity among Portuguese *O. ficus-indica* ecotypes. However, genetic variability in plants is essential for genetic improvement by providing options for the breeders to develop new cultivars. Therefore, the introduction of germplasm and landraces from the centres of origin and domestication, in addition to selected spineless genotypes from other regions, are required to increase the genetic variability of the Portuguese *O. ficus-indica* populations.

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7. Final considerations

7.1 Concluding remarks and future perspectives

Climate change is expected to deeply affect the Mediterranean region, particularly inland areas, in the near future. The cactus pear by its morpho-physiological characteristics and multiple economic uses, represent an alternative crop in the Mediterranean region.

The present work contributed to the characterization and evaluation of Portuguese *Opuntia* spp. germplasm established in a provenance trial at Escola Superior Agrária de Castelo Branco (ESACB), Portugal. The *Opuntia* spp. ecotypes were evaluated for biomass production and nutritional quality of the cladodes when used for fodder, and for fruit yield and quality. In addition, its genetic diversity was assessed by nuclear microsatellite (nuSSR) markers.

We have developed linear models to estimate the *Opuntia ficus-indica* (OFI) cladodes photosynthetic area, the fresh and dry matter production by a non-destructive method. Significant variability in biomass production among the studied populations of OFI was found in this study. Within the set of evaluated Portuguese OFI ecotypes it was possible to select a smal group of spineless ecotypes (OFI-05, OFI-12 OFI-13 and OFI-14) with similar biomass production to the "Gialla" cultivar. Among the selected ecotypes, the population OFI-12 was the most suitable for feeding ruminants. The cladodes of this ecotype are spineless and have higher CP levels when compared to the other Portuguese ecotypes and cv. "Gialla". The OFI can be used for feeding small ruminants provided that animals have access to dry forage and a feed source with high CP content. Used as fodder, *O. ficus-indica* seems to be an interesting feed option for small ruminants in driest period of the year, when there is low quality and quantity of pasture. Therefore, further studies are need to evaluate the performance and nutrient digestibility of small ruminants fed with increasing levels of cactus pear. In regions were the main purpose of cactus is fruit production, cactus forage may be an important pruning by product, helping to feed the livestock, as a replacement of other forages, in inland areas of Portugal.

Regarding he fruit production, the Italian cultivars "Gialla" and "Bianca" clearly outperformed the Portuguese ecotypes, in the second and the third years after plantation, reflecting their origin as improved plant material. Among the 16 Portuguese OFI populations, a variation in fruit yields and fruit distribution across two weight categories was found. The OFI is a valid crop for marginal soils and could be cultivated in a non-tillage system for fruit production, provided that high yield cultivars and appropriate agronomic practices, i.e. pruning, fruit thinning, fertilization and irrigation are used. The major factor limiting the horticultural potential of cactus pear is the poor economic value of its fruits, which, although appreciated in rural communities, still fail to appeal to the urban consumers (Inglese *et al.*, 2002).

Marketing and promotion campaigns are needed, as well as consumer-education strategies at local and international level (Caplan, 1990). Furthermore, constrains to cactus pear consumption are the presence of glochids and hard seeds in the fruit. The obtention of glochid-free cultivars and the reduction of the seed content are challenges for plant breeding (Inglese *et al.*, 2002). The main difficulties in cactus pear breeding are related with the long-term juvenility and the biological complexity namely the polyploidy and the occurrence of apomixis (Mondragón-Jacobo and Chessa, 2017).

The germplasm characterization and evaluation is important since plant genetic resources play an important role in the cultivated plants improvement as a source of genetic variability. However, in countries new to cactus cultivation, as is Portugal, it is recommended to introduce selected *O. ficus-indica* spineless genotypes, test them and propagate the best performers under local conditions before launching large-scale cultivation programmes (Mondragón-Jacobo and Chessa, 2017).

A considerable genetic variation in the concentration of bioactive compounds and morphological characteristics of the fruits was observed among the different *Opuntia* species and among the different OFI ecotypes. The red pulp cv. “Rossa” had the highest betalain content among the *O. ficus-indica* populations, followed by the orange and the white pulp ecotypes. The highest amount of total phenolic compounds was found in the white pulp *O. ficus-indica* ecotypes. The *Opuntia ficus-indica* orange pulp fruits were larger, heavier and had a higher percentage of pulp as well as a lower percentage of seeds compared to the white pulp fruits. Besides, the spineless ecotypes that showed the highest biomass production, as previously mentioned, belong to the group of populations with orange pulp fruits. Therefore, they constitute an interesting source to initiate a breeding program through clonal selection, either for fodder and/or fruit production. The ecotype OFI-04 was distinguishable from the others due to the quantitative and qualitative characteristics of its fruit, and it could be considered a new variety. The *Opuntia*. spp. are an interesting source of phenolic compounds, betalains, and ascorbic acid and the moderate consumption of cactus pear fresh fruit can provide important antioxidant intake.

The genetic diversity of 19 Portuguese *Opuntia* spp. ecotypes, belonging to the species *O. ficus-indica*, *O. elata*, *O. dillenii* and *O. robusta* and three Italian cultivars was assessed using 6 nuSSR markers. Unique microsatellite pattern was found for the different studied species, *O. ficus-indica*, *O. robusta*, *O. dillenii* and *O. elata*, allowing a clearly differentiation at this level. However, at the intraspecific level, only two subclusters were differentiated among *O. ficus-indica* populations and no unique microsatellite pattern was found for each individual accessions, suggesting that some of them could be recent vegetative clones. One subcluster contained the white pulp fruits (including cv. “Bianca”) and the ecotype OFI-

01 (which corresponds to the spinescent form *amyclaea*), and the other contained the orange pulp fruits (including cv. “Gialla”), the cv. “Rossa”, and one ecotype with pale yellow pulp (OFI-04). The spinescent *O. ficus-indica* f. *amyclaea* (OFI-01) did not differ from the *O. ficus-indica* f*. ficus-indica* spineless ecotypes and the clustering did not correspond to spinescence. Although some phenotypic variability was found, for example in differences in vegetative vigor, flower color, cladode form, and the number and presence or absence of spines, the nuSSR indicate a narrow genetic variability among the Portuguese *O. ficus-indica* ecotypes. Recent advances, such as next-generation sequencing, will permit the development of non-neutral markers by targeting genetic regions that are directly influenced by natural selection (Kirk and Freeland, 2011).

The large octoploid genome (4 Gbp) of *O. ficus-indica* makes comprehensive and accurate *de novo* genome sequencing difficult. Multiple ‘omics’ resources are in development for *O. ficus-indica* to enable the identification of key genetic determinantes for adaptations including CAM, tissue succulence, and epicuticular wax synthesis. The *Opuntia cochenillifera* diploid genome is currently undergoing PacBio SMRT sequencing and will increase the assembly of an existing data set from *O. ficus-indica* (Mayer *et al.*, 2016).

In the Mediterranean Basin, some *Opuntia* spp. (mainly *Opuntia ficus-indica* Mill.) were introduced five centuries ago from the areas of origin (Casas and Barbera, 2002) and cactus pear proved to be so well-suited to the environmental conditions that they quickly became naturalized. There is a conflict of interest between those who consider *O. ficus-indica* as an opportunity for agriculture, especially in the inland areas of Portugal, and those who consider it an invasive plant. *Opuntia ficus-indica* became invasive in areas with a wet season characterized by high temperatures, for example, in South Africa and Australia (Zimmermann *et al.*, 2009). In Mediterranean climates, natural invasion is limited by the humidity and cold winter temperatures that contrast the warm, dry conditions of summer (Barbera, 1995). In the Iberian Peninsula *O. ficus-indica* is rarely expanded by seed germination due to thermal and hygrometric conditions, which are seldom optimal for reproduction (Nieddu and Chessa, 1997: Blasco *et al.*, 2015). The *Opuntia ficus-indica* areas of occurrence are related to human activity and its propagation is maintained by asexual reproduction. However, in some areas, its abundant presence occurs in places with steep slopes where the cladodes break off, easily root and form conspicuous patches (Erre *et al.*, 2009). Therefore, the OFI planting in these areas should be discouraged although in some situations OFI has an important role in the soil erosion mitigation (Louhaichi *et al.*, 2017). According to Blasco *et al.* (2015), the spineless cactus pear, *O. ficus-indica* f. *ficus-indica*, never behaves as invasive species because it only forms isolated micro-populations, in the Iberian Peninsula. Additionally, it does not compete with native plants or cause

genetic contamination. The cactus pear could not be considered as potentially invasive plant because its distribution is restricted to cultivated land. The spinescent variety, *O. ficus-indica* f. *amyclaea*, may give rise to little populational nuclei without continuity. Nevertheless, in other countries with Mediterranean climate (island principally), the spiny form could give rise to continuous populations.

The Iberian Peninsula is likely a source of additional morphological and genetic variability inside this genus, and further germplasm collection as well as harvest and subsequent characterization of ecotypes (particularly *Opuntia ficus-indica* f. *ficus-indica*) should be undertaken to better understand the *Opuntia* spp. in this area. Neverthless, in recent years, the cochineal (*Dactylopius coccus*) has been rapidly destroying the *O. ficus-indica* naturalized populations in southern Spain (Cañas, 2017), which represents probably an important loss of genetic variability. Unfortunately, we anticipate the almost inevitable spread of this insect to Portugal, which should deserve the greater attention of the official entities and cactus pear producers to contain this plague.

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8. Appendices

Appendix 1 – Table S2.1

Table S2.1 List of the models to predict the variables *CA*, the *FW* and the *DW*.

Model	Mathematical model	n	a (SE)	b (SE)	R^2	RMSE
1	$CA = a + b\,(L + W)$	60	-330.61 (78.52)	16.01 (1.37)	0.70	47.65
2	$CA = a + b\,(L \times W)$	60	48.13 (22.92)	0.75 (0.03)	0.91	26.68
3	$CA = a + b\,(L + W)^2$	60	128.83 (38.98)	0.14 (0.01)	0.71	47.16
4	$CA = a + b\sqrt{(L \times W)}$	60	-507.78 (46.56)	40.95 (1.74)	0.90	26.89
5	$CA = a + b\sqrt{(L + W)}$	60	-1247.84 (158.16)	241.53 (20.89)	0.70	47.97
6	$FW = a + b\,L$	60	-161.80 (126.62)	27.35 (3.26)	0.55	117.22
7	$FW = a + b\,(L + W)$	60	-902.93 (163.28)	31.36 (2.84)	0.68	99.09
8	$FW = a + b\,(L \times W)$	60	135.81 (111.03)	1.06 (0.15)	0.45	129.25
9	$FW = a + b\,(L + W + T)$	60	-980.39 (156.67)	31.67 (2.64)	0.71	93.50
10	$FW = a + b\,(L \times W \times T)$	60	36.91 (35.97)	0.64 (0.03)	0.91	52.27
11	$FW = a + b\,(L \times W \times D)$	60	289.62 (75.94)	0.25 (0.03)	0.53	119.32
12	$FW = a + b\,(L \times W \times T \times D)$	60	217.40 (29.26)	0.15 (0.01)	0.91	53.20
13	$FW = a + b\,(L + W + T + D)$	60	-1036.98 (156.69)	30.88 (2.50)	0.73	91.54
14	$FW = a + b\,L^2$	60	369.94 (64.52)	0.35 (0.04)	0.55	117.54
15	$FW = a + b\,(L^2 + W^2)$	60	153.45 (72.28)	0.40 (0.04)	0.65	102.95
16	$FW = a + b\,(L + W)^2$	60	3.47 (82.41)	0.27 (0.03)	0.67	99.69
17	$FW = a + b\,(L^2 + W^2 + T^2)$	60	152.45 (72.21)	0.40 (0.04)	0.653	102.80
18	$FW = a + b\sqrt{L}$	60	-1224.19 (252.76)	341.51 (40.68)	0.55	117.24
19	$FW = a + b\sqrt{T}$	60	-3140.93 (290.23)	2680.95 (192.70)	0.77	83.79
20	$FW = a + b\sqrt{(L \times W)}$	60	-662.97 (222.05)	58.26 (8.29)	0.46	128.21
21	$FW = a + b\sqrt{(L + W)}$	60	-2711.54 (326.40)	476.65 (43.12)	0.68	98.99
22	$FW = a + b\sqrt{(L + W + T)}$	60	-2863.59 (313.32)	488.85 (40.73)	0.71	93.49
23	$FW = a + b\sqrt{(L \times W \times T)}$	60	-830.53 (71.07)	47.43 (1.95)	0.91	52.04
24	$DW = a + b\,(L + W)$	60	-22.77 (15.72)	1.55 (0.27)	0.36	9.54
25	$DW = a + b\,(L \times W)$	60	18.18 (7.98)	0.07 (0.01)	0.39	9.29
26	$DW = a + b\,(L \times W \times D)$	59	25.64 (5.16)	0.02 (0.00)	0.53	8.02
27	$DW = a + b\,(L + W + T + D)$	60	-29.43 (16.01)	1.53 (0.26)	0.38	9.35
28	$DW = a + b\,(W \times T \times D)$	58	8.49 (4.79)	0.49 (0.04)	0.72	6.05
29	$DW = a + b\,(W \times T \times D)^2$	58	37.07 (2.48)	0.00 (0.0)	0.73	6.03
30	$DW = a + b\sqrt{(W \times T \times D)}$	58	-48.03 (9.58)	10.58 (0.89)	0.72	6.12

CA – cladode area, one face (cm^2); FW – cladode fresh weight (g); DW – cladode dry weight (g); *L* – cladode length (cm); *W* – cladode maximum width (cm); RMSE – root mean square error; SE – standard error.

Appendix 2 – Figure S2.1

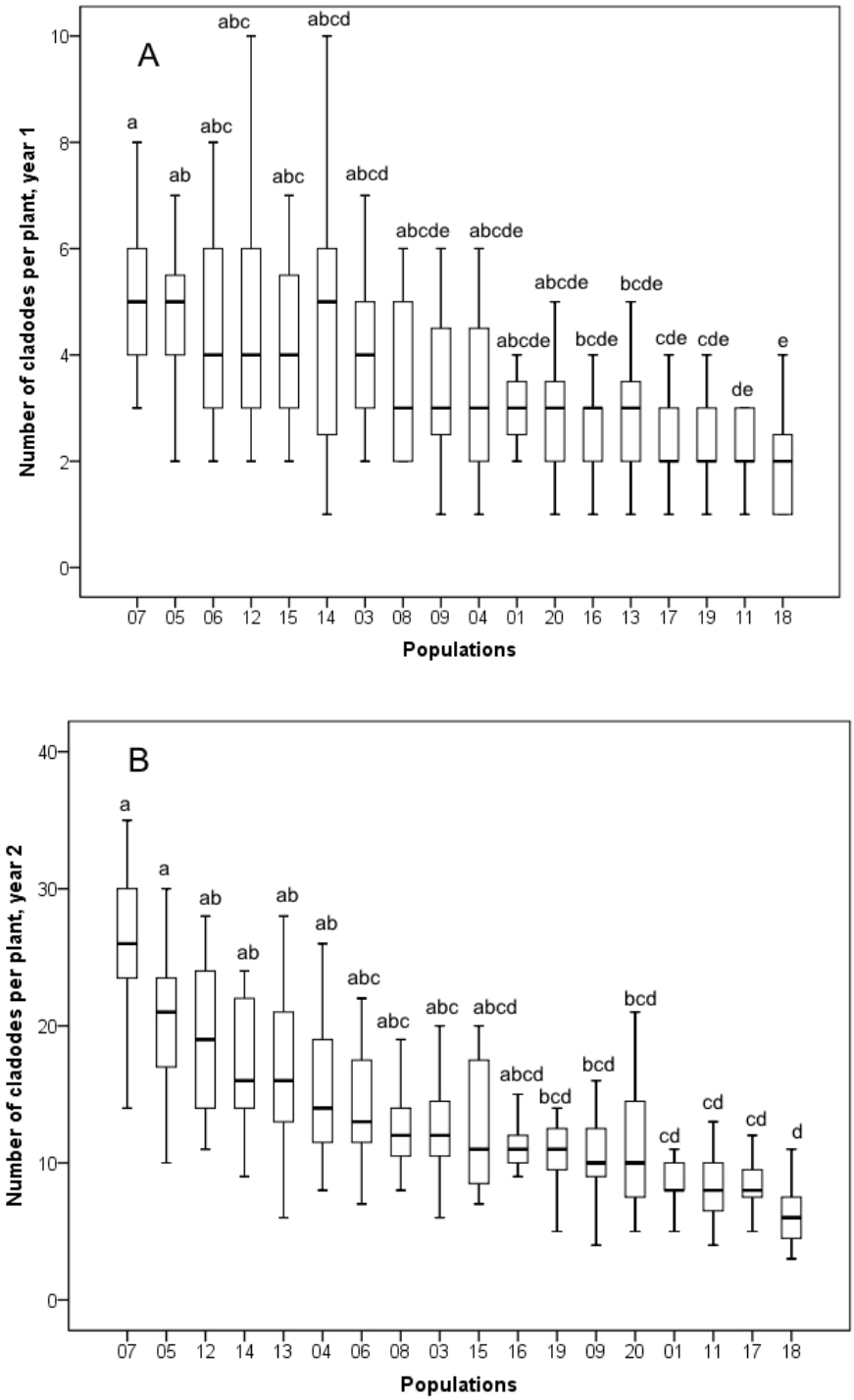

Figure S2.1. Cladode number per plant in year 1 (A, 330 DAP) and in year 2 (B, 660 DAP). Populations with the same letter do not differ according to the Kruskal-Wallis test, followed by multiple comparisons of mean orders for α = 0.05. The bold line represents the median, framed between the 1st quartile (lower end of the box) and the 3rd quartile (upper end of the box). The upper and lower bars represent respectively the minimum and maximum.

Appendix 3 – Table S2.2

Table S2.2 Games-Howell post hoc results for the area of cladodes per plant in year 2.

		Mean Differences (*Xi* - *Xj*)															
Pop	Mean	01	03	04	05	06	07	08	09	11	12	13	14	15	16	17	18	19
OFI-01	0.63																	
OFI-03	1.03	**0.39***																
OFI-04	1.23	**0.59***	0.20															
OFI-05	1.60	**0.96***	**0.57***	0.37														
OFI-06B	1.14	**0.51***	0.12	0.08	0.45													
OFI-07G	1.90	**1.26***	**0.87***	**0.67***	0.30	**0.75***												
OFI-08	0.94	0.30	0.09	0.29	**0.66***	0.21	**0.96***											
OFI-09	0.78	0.14	0.25	0.45	**0.81***	0.37	**1.12***	0.16										
OFI-11	0.56	0.08	**0.47***	**0.67***	**1.04***	**0.59***	**1.34***	**0.38***	**0.22***									
OFI-12	1.59	**0.95***	0.56	0.36	0.01	0.44	0.31	**0.65***	**0.81***	**1.03***								
OFI-13	1.41	**0.77***	0.38	0.18	0.19	0.26	0.49	0.47	0.63	**0.85***	0.18							
OFI-14	1.49	**0.85***	0.46	0.26	0.11	0.34	0.41	**0.55***	**0.71***	**0.93***	0.10	0.08						
OFI-15	0.89	0.25	0.14	0.34	**0.71***	0.26	**1,01***	0.05	0.11	0.33	**0.70***	0.52	**0.60***					
OFI-16	0.84	0.20	0.19	0.39	**0.76***	0.30	**1.06***	0.10	0.06	**0.28***	**0.75***	0.57	**0.65***	0.05				
OFI-17	0.60	0.04	**0.43***	**0.63***	**1.00***	**0.55***	**1.30***	**0.34***	0.18	0.04	**0.99***	**0.81***	**0.89***	0.29	0.24			
OFI-18	0.40	**0.23***	**0.63***	**0.83***	**1.19***	**0.74***	**1.50***	**0.53***	**0.38***	0.16	**1.19***	**1.01***	**1.08***	**0.48***	**0.44***	0.19		
OFI-19	0.76	0.12	0.27	0.47	**0.84***	0.38	**1.14***	0.18	0.02	**0.20***	**0.83***	**0.65***	**0.73***	0.13	0.08	0.16	**0.36***	
OFI-20	0.74	0.11	0.29	0.49	**0.86***	0.40	**1.16***	0.20	0.04	0.18	**0.85***	**0.67***	**0.74***	0.15	0.10	0.15	0.34	0.02

*$p < 0.05$; B – cv. “Bianca”; G – cv. “Gialla”; Pop – Population.

Appendix 4 – Table S2.3

Table S2.3 Games-Howell post hoc results for fresh weight per plant in year 2

		Mean Differences (*Xi* - *Xj*)																
Pop	Mean	01	03	04	05	06	07	08	09	11	12	13	14	15	16	17	18	19
OFI-01	5.54																	
OFI-03	9.52	**3.97***																
OFI-04	9.61	**4.07***	0.10															
OFI-05	11.95	**6.40***	2.43	2.33														
OFI-06B	11.34	**5.79***	1.82	1.73	0.61													
OFI-07G	14.16	**8.62***	**4.65***	4.55	2.22	2.82												
OFI-08	9.50	**3.95***	0.02	0.12	2.45	1.85	**4.67***											
OFI-09	6.86	1.31	2.66	2.76	**5.09***	4.48	**7.30***	2.64										
OFI-11	5.02	0.52	**4.49***	**4.59***	**6.92***	**6.32***	**9.14***	**4.47***	1.83									
OFI-12	12.30	**6.76***	2.79	2.69	0.36	0.96	1.86	2.81	**5.45***	**7.28***								
OFI-13	11.14	**5.60***	1.62	1.53	0.81	0.20	3.02	1.65	4.28	**6.12***	1.16							
OFI-14	12.45	**6.90***	2.93	2.83	0.50	1.10	1.72	2.95	**5.59***	**7.42***	0.14	1.30						
OFI-15	8.61	3.07	0.91	1.00	3.33	2.73	**5.55***	0.88	1.75	3.59	3.69	2.53	3.83					
OFI-16	7.83	**2.28***	1.69	1.79	**4.12***	3.51	**6.33***	1.67	0.97	**2.80***	4.48	3.31	**4.62***	0.78				
OFI-17	6.01	0.46	3.51	3.61	**5.94***	**5.33***	**8.16***	3.49	0.85	0.98	**6.30***	**5.13***	**6.44***	2.60	1.82			
OFI-18	4.19	1.35	**5.32***	**5.42***	**7.76***	**7.15***	**9.97***	**5.30***	2.67	0.83	**8.11***	**6.95***	**8.25***	**4.42***	**3.64***	1.81		
OFI-19	7.19	1.64	2.33	2.43	**4.76***	4.16	**6.98***	2.31	0.33	2.16	5.12	3.96	**5.26***	1.43	0.64	1.18	2.99	
OFI-20	6.03	0.49	3.49	3.58	**5.92***	**5.31***	**8.13***	3.46	0.83	1.01	**6.27***	**5.11***	**6.41***	2.58	1.80	0.02	1.84	1.15

*p <0.05; B – cv. “Bianca”; G – cv. “Gialla”; Pop - Population.

Appendix 5 – Table S2.4

Table S2.4 Games-Howell post hoc results for dry weight per plant in year 2.

		Mean Differences (X i - X j)																
Pop	M	01	03	04	05	06	07	08	09	11	12	13	14	15	16	17	18	19
OFI-01	0.53																	
OFI-03	0.87	0.34																
OFI-04	0.97	**0.44***	0.10															
OFI-05	1.20	**0.68***	0.34	0.23														
OFI-06B	0.96	**0.43***	0.09	0.01	0.24													
OFI-07G	1.45	**0.92***	**0.58***	0.48	0.24	**0.49***												
OFI-08	0.90	0.37	0.03	0.07	0.31	0.06	**0.55***											
OFI-09	0.63	0.11	0.24	0.34	**0.57***	0.33	**0.81***	0.27										
OFI-11	0.48	0.04	**0.39***	**0.49***	**0.72***	**0.48***	**0.96***	**0.42***	0.15									
OFI-12	1.24	**0.71***	0.37	0.27	0.04	0.28	0.21	0.34	**0.61***	**0.76***								
OFI-13	1.09	**0.56***	0.22	0.12	0.11	0.13	0.36	0.19	0.46	**0.61***	0.15							
OFI-14	1.27	**0.74***	0.40	0.30	0.07	0.31	0.18	0.37	**0.64***	**0.79***	0.03	0.18						
OFI-15	0.84	0.31	0.03	0.13	0.37	0.12	**0.61***	0.06	0.20	0.35	0.40	0.25	0.43					
OFI-16	0.72	0.19	0.15	0.25	**0.48***	0.24	**0.72***	0.18	0.09	**0.24***	0.52	0.37	**0.55***	0.12				
OFI-17	0.59	0.06	0.28	0.38	**0.61***	**0.37**	**0.86***	0.31	0.04	0.11	**0.65***	**0.50***	**0.68***	0.25	0.13			
OFI-18	0.43	0.09	**0.53***	**0.53***	**0.77***	**0.52***	**1.01***	**0.46***	0.20	0.05	**0.80***	**0.65***	**0.83***	**0.40***	**0.29***	0.15		
OFI-19	0.68	0.16	0.18	0.28	**0.52***	0.27	**0.76***	0.21	0.05	0.20	0.55	0.40	**0.58***	0.15	0.04	0.10	0.25	
OFI-20	0.66	0.13	0.21	0.31	**0.54***	0.30	**0.78***	0.24	0.03	0.18	**0.58***	**0.43***	**0.61***	0.18	0.06	0.07	0.23	0.02

*p <0.05; B – cv. “Bianca”; G – cv. “Gialla”; Pop - Population.

Appendix 6 – Table S6.1

Table S6.1 Dice similarity coefficients

OFI01	OFI03	OFI04	OFI05	Bianca	Gialla	OFI08	OFI09	*O.dillenii*	OFI11	OFI12	OFI13	OFI14	OFI15	OFI16	OFI17	OFI18	OFI19	OFI20	*O.elata*	Rossa	*O.robusta*	
1.000																						OFI01
1.000	1.000																					OFI03
0.708	0.708	1.000																				OFI04
0.708	0.708	1.000	1.000																			OFI05
1.000	1.000	0.708	0.708	1.000																		Bianca
0.708	0.708	1.000	1.000	0.708	1.000																	Gialla
1.000	1.000	0.708	0.708	1.000	0.708	1.000																OFI08
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000															OFI09
0.381	0.381	0.368	0.368	0.381	0.368	0.381	0.381	1.000														*O.dillenii*
1.000	1.000	0.708	0.708	1.000	0.708	0.708	1.000	0.381	1.000													OFI11
0.708	0.708	1.000	1.000	0.708	1.000	0.708	0.708	0.368	0.708	1.000												OFI12
0.708	0.708	1.000	1.000	0.708	1.000	0.708	0.708	0.368	0.708	1.000	1.000											OFI13
0.708	0.708	1.000	1.000	0.708	1.000	0.708	0.708	0.368	0.708	1.000	1.000	1.000										OFI14
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000	0.381	1.000	0.708	0.708	0.708	1.000									OFI15
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000	0.381	1.000	0.708	0.708	0.708	1.000	1.000								OFI16
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000	0.381	1.000	0.708	0.708	0.708	1.000	1.000	1.000							OFI17
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000	0.381	1.000	0.708	0.708	0.708	1.000	1.000	1.000	1.000						OFI18
1.000	1.000	0.708	0.708	1.000	0.708	1.000	1.000	0.381	1.000	0.708	0.708	0.708	1.000	1.000	1.000	1.000	1.000					OFI19
0.708	0.708	1.000	1.000	0.708	1.000	0.708	0.708	0.368	0.708	1.000	1.000	1.000	0.708	0.708	0.708	0.708	0.708	1.000				OFI20
0.255	0.255	0.326	0.326	0.255	0.326	0.255	0.255	0.378	0.255	0.326	0.326	0.326	0.255	0.255	0.255	0.255	0.255	0.326	1.000			*O.elata*
0.708	0.708	1.000	1.000	0.708	1.000	0.708	0.708	0.368	0.708	1.000	1.000	1.000	0.708	0.708	0.708	0.708	0.708	1.000	0.326	1.000		Rossa
0.372	0.372	0.410	0.410	0.372	0.410	0.372	0.372	0.192	0.372	0.410	0.410	0.410	0.372	0.372	0.372	0.372	0.372	0.410	0.316	0.410	1.000	*O.robusta*

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