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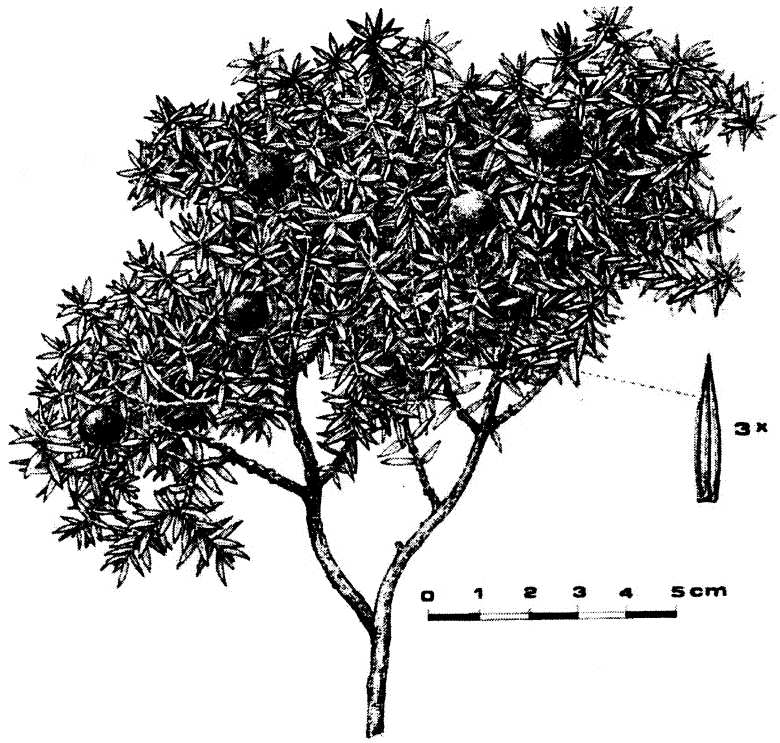
Juniperus navicularis Gand. – Contributos para a sua conservação.



Mário Rui da Costa Basílio e Castro

Dissertação apresentada para a obtenção do grau de mestre em Biologia da Conservação.

Orientador: Prof.ª Drª Amely Zavattieri

Co-orientador: Prof.ª Drª Anabela Belo

Évora, 2009

Esta dissertação não inclui as críticas e sugestões feitas pelo Júri.

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Bem Hajam!

INDICE GERAL

Juniperus navicularis Gand. – Contributos para a sua conservação.

Resumo

Juniperus navicularis Gand. (piorro) é uma conífera endémica portuguesa que constitui frequentemente sub-bosque dos pinhais nas areias de transição de pliocénicas para plistocénicas da região litoral a sul do Tejo.

O objectivo deste trabalho é verificar as localizações documentadas em herbário para esta espécie, avaliando o estado de conservação através do estudo das populações, assim como estabelecer um protocolo laboratorial para a multiplicação *in vitro*.

Das 12 populações identificadas, amostraram-se 4 com distintas características em termos de conservação. Medições espaciais e morfológicas dos indivíduos foram realizadas *in situ*. Realizaram-se ensaios de germinação de sementes e foram testadas diversas combinações meio/hormona em multiplicação e enraizamento.

Diferentes estados de conservação resultam em diferentes morfótipos individuais, tanto ao nível da estrutura como em termos de distribuição sexual e recrutamento de novas plantas.

Apesar das dificuldades no enraizamento, foram multiplicados rebentos desta espécie *in vitro* obtendo-se taxas satisfatórias para a conservação artificial da espécie.

Palavras-chave: população fragmentada; endémica; espécie ameaçada; vigor populacional; micropropagação; Cupressaceae

Juniperus navicularis Gand. – Contributes for its conservation.

Abstract

Juniperus navicularis Gand. (piorro) is a Portuguese endemic conifer that constitutes the understory of pine forests in the plisto-pliocénic transition sands of the seaside region of SW Portugal.

The main objective of this work is to verify documented locations in national herbariums, examining its conservation status through individual studies, as well as establishing a laboratorial protocol for *in vitro* multiplication.

From the 12 identified populations, 4 were sampled for their distinct characteristics in terms of conservation status. Spatial and morphological measurements were made *in situ*. Seed germination essays were made and several medium/hormone combinations were tested in plant multiplication and rooting.

Different conservation degrees result in visible differences on individual morphotypes, both structurally and in terms of sexual distribution and new plants recruitment.

Despite the rooting difficulties, juniper shoots were multiplied *in vitro*, achieving quite satisfactory rates for the artificial conservation of this particular species.

Key-words: fragmented population; endemic; threatened species; population fitness; micropropagation; Cupressaceae

Introdução

A biodiversidade mundial está a decrescer a um ritmo sem precedentes. Entre 1996 e 2008, cerca de 8500 espécies de plantas foram adicionadas ao Lista Vermelha de Espécies Ameaçadas da International Union for the Conservation of Nature (IUCN, 2009). O principal propósito da Lista Vermelha da IUCN é catalogar e evidenciar aqueles taxa que apresentam um risco maior de extinção global (ex.: listados como criticamente em perigo, em perigo e vulneráveis). Durante este período, houve também um aumento de quase 75% no número de plantas assinaladas como criticamente em perigo (IUCN, 2009). Estes valores são alarmantes e são necessárias medidas imediatas de conservação para salvaguardar muitas destas espécies.

O *Juniperus navicularis* Gand. (= *J. oxycedrus* L. subsp. *transtagana* Franco), zimbro-galego ou piorro, é um arbusto fatigiado dióico da família das Cupressaceae. É uma espécie que ocorre nas areias marítimas, nomeadamente em dunas costeiras do Plistocénio, maioritariamente no Distrito de Setúbal (estuário do Rio Sado) a sudoeste de Portugal continental (Rivas-Martínez *et al.*, 1990) e crê-se que seja um endemismo português. Apesar de haver alguns registos da ocorrência desta espécie em Espanha (Franco e Rocha, 1982) estas informações requerem confirmação. Esta espécie pertence às formações xerofíticas da seca área bioclimática psamofila termo-mediterrânica da costa sul e oeste, claramente caracterizadas por outros arbustos esclerófilos como: *Rhamnus oleoides* subsp. *oleoides*, *Pistacia lentiscus*, *Myrtus communis* e *Quercus coccifera* subsp. *coccifera* (ICN, 2005).

O *J. navicularis* tem maioritáriamente como seu habitat regosolos ácidos ou arenosos com pouca disponibilidade de nutrientes e uma baixa capacidade hídrica, não influenciada por aquíferos subterrâneos, já que ocupa o topo das Paleo-dunas Plistocénicas (dunas consolidadas). As áreas onde ocorre, mais precisamente na bacia quaternária do Sado, de onde é endémico, apresentam algumas populações bem conservadas, especialmente em zonas com altas densidades de pinhal (Costa et al, 1993), ou em áreas onde as técnicas de corte e arroteia de arbustos foram abandonadas. Apesar disso, o *J. navicularis* é uma espécie ameaçada devido ao desmesurado crescimento urbano nas áreas de onde ele é nativo. Juntamente com a fragmentação de zonas costeiras outrora pristinas, em parte por ocupação para fins turísticos, as técnicas não selectivas utilizadas nos tratamentos a que o seu habitat é normalmente submetido,

quer com o intuito de redução da proliferação de arbustos como para reduzir o risco de incêndio, são a principal causa de redução das suas populações de crescimento lento (ICN, 2005), podendo levar a uma série de anos sem que torne a ocorrer recrutamento de novos indivíduos para a população.

Esta espécie é extremamente importante em associação com o *Pinus pinea,* dado que tem a capacidade para contribuir na regulação tanto do ciclo hídrico como dos nutrientes nestes solos tão particulares, assim como criando as condições essenciais para o refugio e alimento de espécies faunísticas das dunas. Não obstante de todas as características já descritas, o *J. navicularis* é também uma planta importante ao nível farmacêutico, já que foi utilizado etnobotanicamente no passado para fins veterinários, e mais recentemente, diversos óleos essenciais foram descritos para as suas folhas (Adams, 1998; Velasco-Negueruela et al., 2002) e bagas (Cavaleiro et al., 2003).

O último estudo populacional para o *J. navicularis* foi feito nos anos 80 por Franco & Rocha Afonso (1982), localizando a área de ocorrência deste endemismo português. Desde então, muitas das áreas assinaladas foram desmatadas para dar origem a comunidades urbanas e expansão industrial, pelo que se torna urgente tomar conhecimento das populações sobreviventes e do seu actual estado de conservação.

Uma outra dificuldade em termos de conservação para esta espécie é um problema comum para o género dos juniperos: a baixa taxa de germinação das suas sementes. Em consonância com a baixa e irregular produção de sementes (Garcia et al., 1998), a maioria dos juniperos apresentam problemas de germinação de sementes (Juan et al., 2006) devido a um comportamento abortivo ou de dormência por parte dos embriões (Garcia et al., 1998). Esta debilidade pode estar relacionada com alterações climáticas, dado que verões mais quentes e secos podem influenciar a viabilidade das sementes por stress hídrico (Garcia et al., 1998). Este tipo de problemas influencia directamente o recrutamento de novos indivíduos, pondo em risco a sobrevivência das suas populações.

A utilização de biotecnologia, como uma ferramenta para a conservação, é uma ideia que tem vindo lentamente a espalhar-se pela comunidade científica, especialmente no que diz respeito à botânica Para além das aplicações óbvias de técnicas como a multiplicação *in vitro* na conservação de espécies ameaçadas, muitas técnicas de propagação e conservação de germoplasma podem ser utilizadas para salvaguardar plantas que apresentam graves problemas de fragmentação de habitat e propagação *in situ* (Sarasan et al., 2006). Contudo, estas técnicas são extremamente específicas para

cada espécie vegetal, necessitando de bastante tempo para se seleccionar as melhores e mais produtivas, e finalmente, melhora-las para taxas aceitáveis.

Assim, e baseado nestes argumentos este trabalho define-se em duas partes:

A primeira, visa localizar populações previamente identificadas e descritas de *J. navicularis*, tanto em colecções de herbários nacionais como em artigos publicados, examinar o seu actual estado de conservação, através de estudos morfológicos e de distribuição espacial dos seus indivíduos e, através de ensaios laboratoriais, verificar a viabilidade geral das sementes desta espécie.

A segunda parte do trabalho foi elaborada com o intuito de compensar as debilidades no recrutamento de novos indivíduos por parte do género Juniperus, procurando-se assim estabelecer e melhorar um protocolo de multiplicação *in vitro*, que permita salvaguardar, *ex situ,* este endemismo nacional.

Studies on population structure and seeds germination of *Juniperus navicularis* Gand.
Rare and endemic species from the Plio-Plistocene transition sands of west coast Portugal

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ABSTRACT

Juniperus navicularis Gand. (Portuguese Prickly Juniper) is a Portuguese dioecious endemic conifer that constitutes the understory of pine forests in the Plio-Plistocene transition sands of SW Portugal seaside regions. Since areas of occurrence of this species are coincident with past and future urban expansion zones, many populations have been lost, and many others are in jeopardy of being destroyed. A population identification and insightful study was performed to evaluate the conservation status of surviving populations. Seed germination essays were carried out in laboratory to fully understand new plant recruitment capabilities of *J. navicularis*. Some of the previous recorded populations were extinct due to urban expansion. Among surviving populations, 3 were chosen to be studied due to their different apparent conservation condition and size. Several differences between populations fitness were detected. Deviations from 1:1 between male and female plants were found. Female plants attained higher values in both height and canopy area for most of the studied populations. Germination trials showed no positive results, with none of the essayed seeds germinating. These results suggest that *J. navicularis* may be facing seed germination problems, which may lead to a reduction of plant variability due to vegetative propagation dominance.

Key words: fragmented population; population fitness; endemic; endangered species;

INTRODUCTION

Juniperus navicularis has its natural habitat mainly in sandy or acid regosols with poor nutrient availability and low water capacity not influenced by the underground water since it occupies the top of the Plio-Plistocene transition sand dunes (consolidated dunes). Endemic to Sado quaternary basin, Portuguese Prickly Juniper has

some well conserved populations, especially in areas with a high density of pine trees (Costa et al, 1993), or in zones were bush-cutting techniques have been abandoned. *Juniperus navicularis* is a threatened species mainly due to the urban expansion in its native areas. Along with fragmentation of once pristine coastal areas caused by human occupation, management of its habitats (*Pinus pinea* or *Pinus pinaster* or mixed stand forest) in order to reduce bush proliferation and prevent fire spreading also poses a threat, if non selective techniques are used, destroying the Portuguese Prickly Juniper slow growing populations (ICN, 2005), what may lead to several non recruiting years. This species is extremely important from an ecological point of view, since it has the capacity to contribute for nutrient and water cycle regulation in these sandy soils and act as a refugee for fauna of dunes (ICN, 2005). *J. navicularis* is also a valuable plant due to its pharmacological interest since it was used in the past for veterinarian purposes and, more recently, several essential oils contained in its leaves (Adams, 1998; Velasco-Negueruela et al., 2002) and berries (Cavaleiro et al., 2003) have been described.

Another conservation issue for this species is a common problem in Juniper genera: the low germination rates of their seeds. Along with low and irregular seed production (Garcia et al., 1998), most junipers have seed germination problems (Juan et al., 2006) due to dormant or abortive behaviour of the embryos (Garcia et al., 1998). This deficiency might be related with climate alterations, since warmer and drier summers can influence seed viability due to water deficit stress (Garcia et al., 1998). Problems with seed production and germination directly influence the recruitment of new individuals and, therefore the survival of populations.

Areas of occurrence of *Juniperus navicularis*, "Littoral dunes of *Juniperus* spp." are protected under the Habitat Directive 92/43/CEE – Annex I and in Portuguese Law nº 140/99 from 24 of April – Annex B-1. More recently, law nº 49/2005 – Annex B-1 also includes this habitat as "Natural and Semi-natural areas" of special interest, therefore allowing their inclusion in ZPE's (Special Protection Zones).

The last study for *J. navicularis* populations was made in the 80's, by Franco & Rocha Afonso (1982), locating and limiting the area of occurrence of this Portuguese endemism. Since then, several areas were razed for urban and industrial expansion. The objective of this work is to locate previously identified *J. navicularis* populations and examine their actual conservation status, through morphological and spatial study of several population individuals. In order to get a new insight on general seed viability of this magnificent endemism, seed germination trials were also performed in laboratory.

MATERIALS AND METHODS

Study Species

The Portuguese Prickly Juniper, *Juniperus navicularis* Gand. (= *J. oxycedrus* L. subsp. *transtagana* Franco) is a fastigiated dioecious shrub of the Cupressaceae family (Franco, 1986). The species inhabits the maritime sands (Plio-plistocene transition sand dunes near the coast) of the Sado District mainly in Sado Estuary (SW Portugal) (Rivas-Martínez *et al.*, 1990) and is thought to be a Portuguese endemism. Allegedly there are some records of this species in Spain (Franco & Rocha Afonso, 1982) but they need to be confirmed. The species belongs to the xerophytic formations of the psammophil dry thermo-mediterranean bioclimatic area of south and west coast characterised also by others sclerophyllous shrubs like: *Rhamnus oleoides* subsp. *oleoides*, *Pistacia lentiscus*, *Myrtus communis* and *Quercus coccifera* subsp. *coccifera* (ICN, 2005).

Information gathering

The population search started crossing information from available bibliography (Fig. 1) on the species with records from national herbariums. In accordance to previous studies, it was then possible to limit the area of study (Annex – Fig.1) Some records are more than 100 years old and the populations that are pointed there have been razed to give space to urban growth. Those records where therefore eliminated, being substituted for locations nearby that still presented some "pristine" covering. Some recent records of *J. navicularis* from the national herbariums presented Global Positioning System (GPS) data. Scientific papers containing information about the location of this species were also considered and, if needed, authors were contacted to obtain further information about the collection sites.

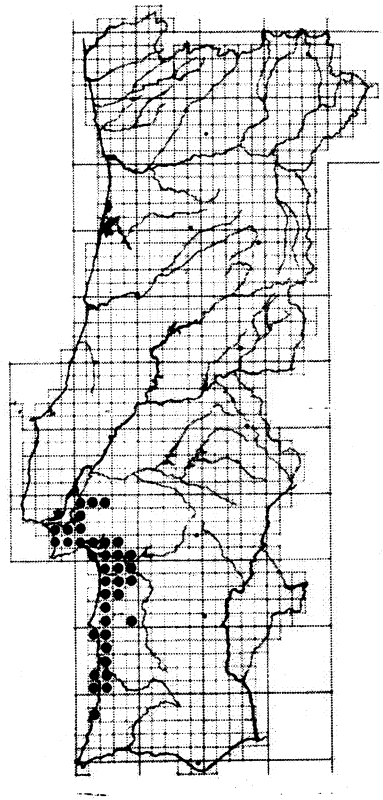

Fig. 1 - Areas of occurrence of *J. navicularis* according to Franco & R. Afonso (1982)

Locating populations

Using GPS data, populations described in herbariums were located by going directly to the coordinates that have been supplied. The same procedure was followed regarding populations that have been mentioned in other studies. Populations from older records, without GPS data, were confirmed by going to the location cited and doing visual inspection on areas of occurrence of *Pinus pinea* and/or *Pinus pinaster*, two species normally associated with *J. navicularis*.

Finally, a study was made using Geographical Information System (GIS) tools as ArcView 3.2, with which was possible to extrapolate several locations where *Juniperus navicularis* could occur. This was done by superposing litological, hydrological and hypsometrical data (IA, 2007) appropriate for this species according to Franco & Rocha Afonso (1982), and Soil Ocupation Charts (COS 90; IGEO, 95). This study had the objective of significantly reducing the search area for *J. navicularis*, eliminating areas that presently have urban tissue instead of forests. Non coherent soil areas (non sandy soils from the Pleistocene era) for this species were also eliminated (Fig. 2).

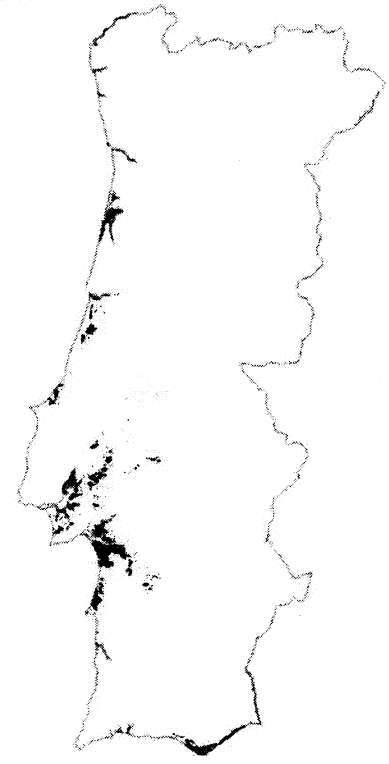

Fig. 2 - Calculated areas of occurrence through GIS processing.

Population study

Population density was calculated by counts of individuals on 16 m^2 squares. Studies were made in situ, using distance sampling techniques (an adaptation of point centered quarter and nearest neighbor methods) along transects of 100 meters. A 50 m long measuring tape was set as guideline for the sampling. After stretching the guideline, ten squares with 16m^2 were marked on the field as a study area, every 10 meters for the total 100. At each square, the following data was recorded: number of individuals inside the quadrat, nearest individual from the 10 meter mark, first and second degree nearest neighbors, and their individual characteristics (sex; height; canopy greater and smaller diameters; sexual maturity).

Fruit and seed collection

In each study site, *Juniperus navicularis* cones were collected from 25 randomly selected female shrubs. Therefore, 20 cones were collected from each individual, summing up 500 juniper cones from each site, in order to study the correlation between size, weight and number of apparently viable seeds in each population. Cone size was measured using an electronic digital caliper, and weight using Ohaus GA200 and Sartorius BP310S analytical balance. Seeds were then extracted, counted and analyzed for parasitic infections.

Germination experiments

Since mature juniper seeds tend to lose viability (Juan et al., 2006), seeds were collected from immature cones, separated into groups and used in several germination experiments (table 1) according to Juan et al. (2006).

Table 1- Germination experiments performed.

Exp.	Number of seeds	Substrate	Treatment	Stratification
1	100	Vermiculite	---	---
2	100	Perlite	---	---
3	100	Vermiculite	0,1 HCl for 2 days	---
4	100	Perlite	0,1 HCl for 2 days	---
5	100	Vermiculite	---	10 days at 4ºC
6	100	Vermiculite	---	90 days at 4ºC
7	50	Agar + Water	---	---

Seeds were sterilized using sodium hypochlorite (3%) during 20 minutes, followed by 3 rinses with bidestiled sterile water. Next, they were placed in 70% alcohol for 2 minutes in agitation, and rinsed three more times with bidestiled sterile water. This was made in sterile conditions so possible germinations could occur with no bacterial of fungal infections. Different substrates were then tested along with different stratification times. Seeds were placed in trays with the substrates and watered frequently enough to keep the substrate wet. The trays were placed in culture chambers and observed every week for new germinations. All experiments took approximately 100 days. Growth chamber conditions were 25ºC/15ºC at a 16/8h day/night period. Experiment 7 was performed in Petri dishes, using only agar as a water supplement media.

Data analysis

Differences between mean plant density, distance between individuals, height and canopy area, for each population, were evaluated using one-way ANOVA followed by LSD post-hoc tests in SPSS v.16. Canopy area was ln transformed to obtain variance homogeneity. The same evaluation was made for weight, width, height and seed content of each population cones. Weight, height and seed content were ln modified prior to analysis to obtain homogeneity of variances.

Spatial distribution of male, female, and juvenile plants was assessed by calculating their respective coefficient of aggregation according to Hopkins (1954) and Welden et al. (1990).

Chi-square statistic test was used to compare the number of male and female plants based on an expected 1:1 ratio of males to females ($P < 0.05$).

RESULTS

Locating populations

The presence of J. navicularis in previously described areas was researched. Most of them disappeared with urbanization and the remaining were usually fragments of once bigger areas. It was possible to locate 3 isolated populations at Fernão Ferro, Pinhal do General and Apostiça, within pine forested areas. Fernão Ferro population was composed of few dwarf individuals, near a waste dumping site. Pinhal do General nucleus is a highly endangered juniper population since it is located between two largely populated urban areas. The survival of this nucleus is therefore intimately connected with the urban management of that area. On the other hand, Apostiça area is better preserved and the populations tend to thrive in pine forest under military management. Nonetheless, and besides the excellent conditions of its population, cleaning of the pine forest using non selective mechanical techniques might put at risk the maturation and spreading of this slow growing species. The other records mentioned the presence of this species in south of Troia Peninsula (as far as Sines) and toward East, along the Sado River until Pinhal de Arez. One population was located next to Troia Peninsula, at Comporta. The individuals were scarce and scattered through a large area. The presence of other populations nearby couldn't be confirmed due to a recent forest cleaning. Farther south, two populations were found near the road. They were

composed of few individuals each and no larger populations could be confirmed in the surroundings. Among the shrubs of Praia do Monte Velho, near Lagoa de Santo André, another small population was found, composed uniquely by dwarf individuals. To the East of Troia, two small populations (less than 20 individuals) and a larger one (more than 500 individuals) was found in Pinhal de Arez. The smaller populations were located near the road, in uncleared areas. The pine forest surrounding these areas was completely cleared of shrubs due to anthropogenic interference for forest maintenance purposes. Pinhal de Arez population was located in private property. The area presented itself lightly disturbed, with new plantations of *Pinus pinea.*

Population Study

Three populations were selected in accordance to habitat fragment dimension and *Pinus* forest density: a small fragment (Pinhal do General) in the middle of a heavily constructed area and two bigger fragment without houses nearby, under a sparse *Pinus* cover (Pinhal de Arez) and a dense one (Apostiça).

Despite the similarities among the three populations studied, several differences can be observed. Number of individuals per study area was approximately the same for Pinhal de Arez and Apostiça populations (table 2) reflecting a lower average distance between plants, when compared to Pinhal do General population where individuals tend to be far more broadened.

Table 2 –Morphological parameters (average values). Values with different letters in the same column are significantly different.

	Density per study area (n=10)	Distance between plants (m) (n=30)	Height of Plants (m) (n=30)	Canopy Area (m^2) (n=30)
Pinhal Arez	7,5 (±1,07) a	1,17 (±0,17) ab	0,68 (±0,05) a	0,39 (±0,06) a
Pinhal General	4,5 (±0,58) b	1,55 (±0,15) a	0,63 (±0,05) b	0,12 (±0,02) b
Apostiça	7,6 (±0,95) a	1,13 (±0,13) b	0,49 (±0,05) c	0,13 (±0,03) b

One-way ANOVA in SPSS v.16 with Post-hoc tests (LSD test); $p< 0,05$

In terms of morphological parameters, Pinhal de Arez and Pinhal do General populations have higher plants than Apostiça, but all are statistically different from another (table 2). Nevertheless, General and Apostiça plants shared identical values for canopy areas, whilst Arez plants canopy area was three times larger, differing significantly from the other two populations. A less pronounced height/canopy area

ratio can also be seen for Arez population (1,74) comparatively with General (5,25) and Apostiça (3,77) populations.

All populations showed different average number of individuals, both for mature and non mature plants (figure 3a). In terms of height by age and sex, female plants tend to be higher in both Pinhal do General and Apostiça populations, but Pinhal de Arez population shows similarly higher plants for male (0,71±0,09) and female shrubs (0,73±0,06) (figure 3b). Pinhal de Arez population has significantly bigger male and non mature shrubs (0,71±0,09 and 0,48±0,09 respectively) but female plants from Pinhal do General are the highest (0,94±0,07). Some variation exists in terms of canopy area, for all populations, both between sex and age of shrubs, but Pinhal de Arez plants also tend to be far more prostrated (0,43±0,07) than the slimmer individuals from P. General (0,16±0,02) and Apostiça (0,16±0,03).

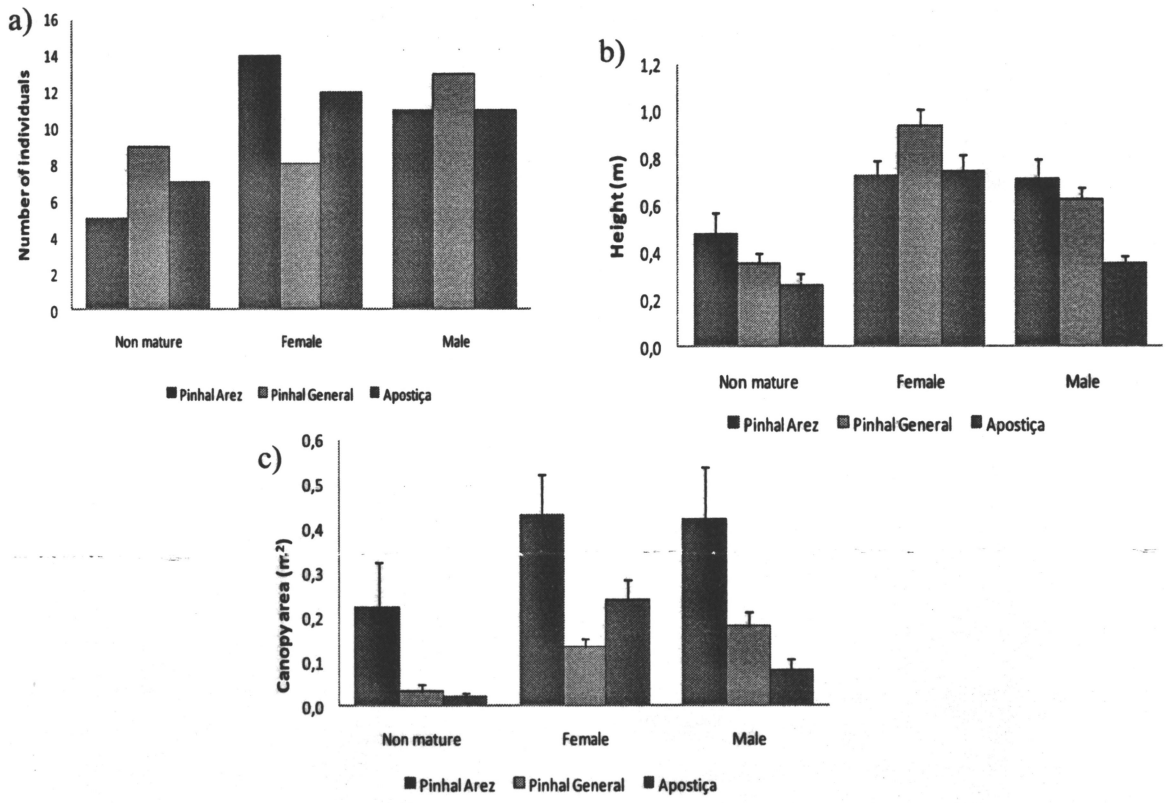

Figure 3 – a) Number of individuals, by sex and age, per population. b) Average height calculated for each population, per sex. c) Average plant canopy area per sex, for each population studied.

Aggregation studies demonstrate that mature populations from Pinhal do General and Apostiça have random distribution, while Pinhal de Arez has an aggregated distribution (table 3). Non mature plants consistently appear more aggregated then

mature ones. These results are in agreement with Gibson & Menges (1994) work with another dioecious shrub.

Table 3 –Hopkins Coefficient of Aggregation (CA) of mature and non mature plants from each population.

Site	Non mature	Mature
Pinhal de Arez	4,13	2,92
Pinhal do General	1,43	1,00
Apostiça	10,25	1,06

Discrepancy between the analyzed population's number of males and females are different from the expected 1:1 ratio only for Pinhal do General (table 4). Number of non mature plants varies between sites. Pinhal do General population had a relatively higher proportion of non matures (30%) compared with Apostiça and Pinhal de Arez (23,3% and 16.7% respectively).

Table 4 – Numbers of *J. navicularis* at the three studied sites. The χ^2 statistic compares the number of male and female plants and is calculated on an expected 1:1 ratio of females to males; $P < 0.001$.

Site	Total	Males	Females	χ^2	Non matures
Pinhal de Arez	30	11	14	1,08	5
Pinhal do General	30	13	8	1,95	9
Apostiça	30	11	12	0,14	7

Fruit and Seed Collection

Through a close analysis of table 5, it is possible to see that all populations have their own cone characteristics. Pinhal do General cones are smaller and lighter than those from the other two populations. Comparatively, Pinhal de Arez cones are not oval shaped as the others, but spheroid and a little heavier. In terms of number of seeds, differences weren't significant among Pinhal de Arez and Pinhal do General. However, Apostiça's *J. navicularis* cones had a significantly greater proportion of seeds than the other populations, probably due to different plant fitness.

Table 5 – Cones morphological parameters and seed numbers (average values). Values with different letters in the same column, are significantly different (n=30).

	Height (mm)	Width (mm)	Weight (g)	Seeds/cone
Pinhal Arez	8,05 (±0,03) a	8,45 (±0,04) a	0,35 (±0,00) a	1,62 (±0,03) a
Pinhal General	7,19 (±0,03) b	6,36 (±0,03) b	0,17 (±0,00) b	1,58 (±0,03) a
Apostiça	8,30 (±0,03) c	7,73 (±0,04) c	0,28 (±0,00) c	2,00 (±0,03) b

One-way ANOVA in SPSS v.16 with Post-hoc tests (LSD test); $p < 0,05$

Germinations Experiments

From the 650 seeds used in the germination essays, none has germinated. For experiments 1 to 6, most of the seeds appeared to be viable, not showing a decaying aspect or colonization by fungi. They were stiff to the touch and fairly hydrated. Experiment 7 allowed the seeds to maintain their primary aspect, avoiding the browning of the other experiments. The enlargement of the seeds due to hydration was visible but no germination was recorded. Bacterial proliferation was visible in two seeds only.

DISCUSSION & CONCLUSION

Most of the populations described in herbarium and in scientific papers were verified in situ (see Annex, Fig.1). The older herbarium records dated from late nineteenth-century and are outdated since most of the areas reported to have *J. navicularis* populations are now part of the urban matrix. Besides the populations found and registered, this species recent area of occurrence is not well known since no census have been made in the past decades.

Many of these previously identified populations of *J. navicularis* have been razed or face imminent destruction due to anthropogenic interference. Those that still persist show different responses to the type of management that their area is submitted. Whilst undisturbed populations tend to be randomly distributed, with a male:female ratio close to 1, populations recovering from non selective cutting are more aggregated and have a more profound sexual deviation, mostly due to vegetative propagation. Nonetheless, highly disturbed areas may also show random distribution, not resulting from individual competition but from the lack of recruitment in the area. A higher proportion of juveniles was also expected for all populations. The highest proportion of juveniles reached 30% for Pinhal do General site, which curiously is the most disturbed area, but most of these juveniles are vegetatively linked to nearby adult plants.

The fact that junipers, as well as other conifers, present several difficulties in seed germination, imply that most of the population individuals reproduce vegetatively, highly diminishing the genetic variability on site. This can also contribute to explain the aggregation patterns and the sexual variation verified. New studies are being undertaken to fully understand *Juniperus* male:female ratio with aggregation patterns.

Populations demonstrate different kinds of responses to habitat maintenance, from the scattering of adult individuals in well conserved areas, to the visible grouping in disturbed populations. These differences have been observed in the genera, and are an obvious response of the species in order to create the microhabitat conditions required for the recruitment of new shrubs (Verdú, 2004), although too much shading can inhibit juvenile growth.

The lack of positive results in the seeding experiments wasn't very surprising because seeds of Juniperus genera usually have germination problems associated with embryo abortion and recalcitrance (Juan et al., 2006; Mamo et al, 2006).

Based on these facts, conservation actions must be undertaken in order to safeguard this unique species. Translocation of plants between nearby populations to slightly improve genetic variability, as well as limiting non selective bush cutting techniques in privileged areas may allow some populations to recover. *Ex vitro* conservation techniques are also an important and reliable tool for preserving endangered species.

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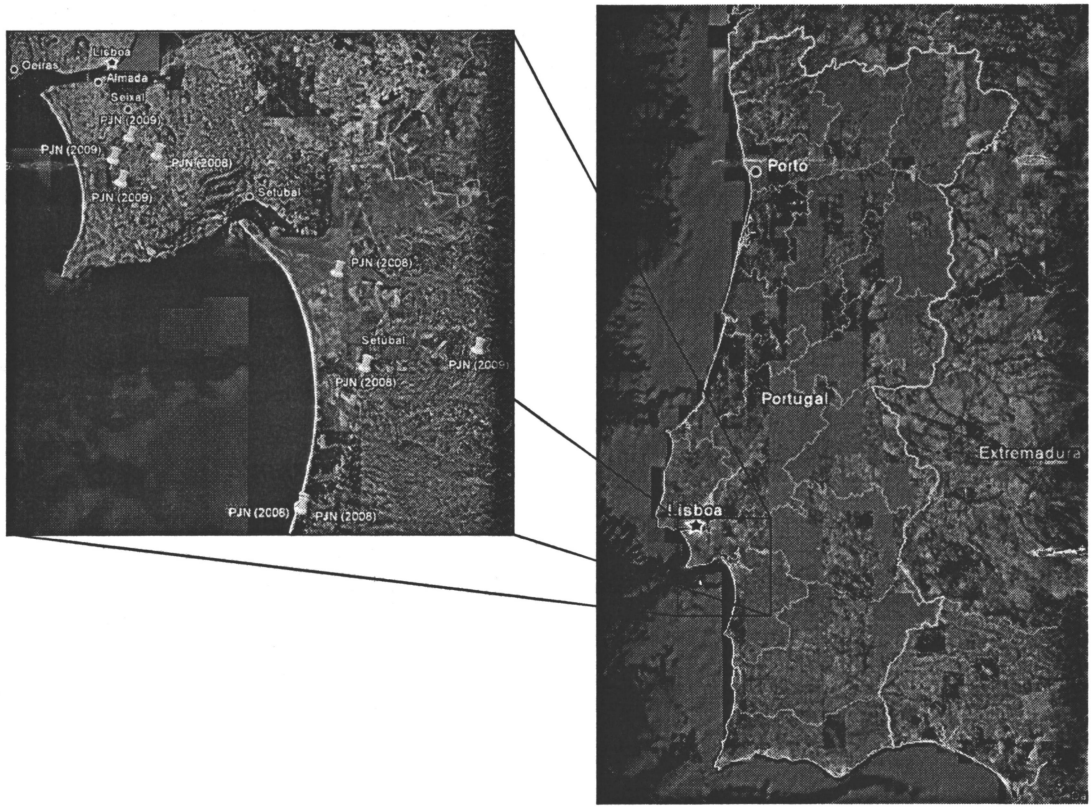

Figure 1: Perspective of the study area in the national context – populations verified in situ.

In vitro multiplication of *Juniperus navicularis* Gand. Rare and endemic species from the Plio-Plistocene transition sands of west coast Portugal

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ABSTRACT

Juniperus navicularis is an endemic shrub that can only be found in a relatively small area of the Portuguese coast line. It grows well in mixed stands with *Pinus pinea* and more rarely with *Quercus lusitanica*, but the non selective techniques used to maintain these woodlands normally involve the removal and destruction of grass and shrub coverage, destroying or damaging *J. n*avicularis populations. Also, the extensive growth of touristic areas, along with a low seed germination rate may endanger even more this most interesting species. Our work focused on safeguarding this unique species using biotechnology i.e., tissue culture. Several culture media were tested for tissue multiplication each containing different plant growth regulators combinations. Three different multiplication cycles were made with diverse evaluation objectives. During the first cycle of multiplication several plant response parameters were analyzed. Olive Medium supplemented with 0,45 μm 6-Benzyl Amino Purine was the most favorable after three multiplication cycles, when compared with other media that produced the best results in the first and second multiplication cycles. Even when different treatments were used for root induction, the media tested failed to induce roots in this species. New rooting experiments are being carried out *in vitro* and *ex vitro.*

Key words: micropropagation; tissue culture; endangered species, Portuguese prickly juniper, Cupressaceae

Abbreviations: AE: Arnold and Eriksson; BAP: 6-benzyl amino purine; BSW: Bidistilled Sterile Water; GD: Gupta and Durzan; KIN: Kinetin; MS: Murashige e Skoog;

NAA: Naphthaleneacetic Acid; OM: Olive medium; PGR: Plant Growth Regulator; SH: Shenk and Hildebrandt; WH: White medium; WPM: Woody Plant Medium;.

INTRODUCTION

The Portuguese prickly juniper or "Piorro", *Juniperus navicularis* Gand. (= *J. oxycedrus* L. subsp. *transtagana* Franco) is a fastigiated shrub from the Cupressaceae family (Fig. 1). The species inhabits the Plio-Plistocene maritime transition sands (dunes near the coast) and is endemic to the Sado District (mainly to Sado River Estuary) of SW Portugal (Rivas-Martínez *et al.*, 1990). It belongs to the xerophytic formations of the psammophil dry thermo-mediterranean bioclimatic area of south and west coast characterised also by the presence of others sclerophyllous shrubs like: *Rhamnus oleoides* subsp. *oleoides*, *R. alaternus*, *Osyris lanceolata* (= *O. quadripartita*), *Pistacia lentiscus*, *Myrtus communis*, *Asparagus aphyllus*, *Phillyrea angustifolia*, *Corema album* (Neto, 2002).

The Portuguese prickly juniper has its natural habitat mainly in sandy or acid regosoils which are poor in nutrients and have low water retention capacity. Moreover the habitat is not influenced by the underground water since it occupies the top of the Plio-Plistocene Paleo-dunes (consolidated dunes). Endemic to the Sado quaternary basin, it has some well conserved populations, especially in areas with a high density of pine trees, or in zones were bush-cutting techniques have been abandoned. The importance of the urgent conservation of *J. navicularis* is mostly due to the urban expansion that can be seen in most of its native areas. Along with the human occupation of once pristine coastal areas, maintenance treatments done to its habitat, the *Pinus pinea and/or Pinus pinaster* forests, also poses as a threat, since non selective techniques are used, which destroy the *J. navicularis* slow growing populations in order to reduce bush proliferation or to prevent fire spreading (ICN, 2005). The species is extremely important in association with *P. pinea* since it has the capacity to contribute for nutrient and water cycle regulation in these particular soils and act as a refuge for fauna of dunes (ICN, 2005). *J. navicularis* is also a valuable plant due to its pharmacological interest since it was used in the past for veterinarian purposes, and most recently, several essential oils contained in its leaves

(Adams, 1998; Velasco-Negueruela et al., 2002) and berries (Cavaleiro et al., 2003) have been described.

Additionally, the low germination rate of seeds is another conservation issue for this species. Along with low and irregular seed production (Garcia, 1998), most junipers have seed germination problems (Juan et al., 2006) due to dormant or abortive behaviour of the embryos (Garcia, 1998). This deficiency might be related with climate alterations, since warmer and drier summers can influence seed viability due to water deficit stress (Garcia, 1998). Problems with seed production and germination directly influence the recruitment of new individuals and, therefore the survival of populations.

Due to the lack of any publication dealing with tissue culture of this species, various tissue culture media, plant growth regulators (PGRs), gelling agents, and the male and female plants were tested to achieve the most satisfactory response at each micropropagation stage i.e., organogenesis initiation, shoot multiplication and rooting.

MATERIALS AND METHODS

Plant material and explant preparation

Different population of *J. navicularis* were identified and characterized along the West Coast of Portugal (Annex Figs. 1 and 2). *In situ* collection of plant material excised from terminal young shoots were collected during October and November 2008 identified by the collection place and transported to the laboratory. *J. navicularis* plants are either male or female so both were identified and used in the cultures (Fig. 3). Shoot tips and nodal segments (no more than 1 cm long), were separated from the collected material and used as explants.

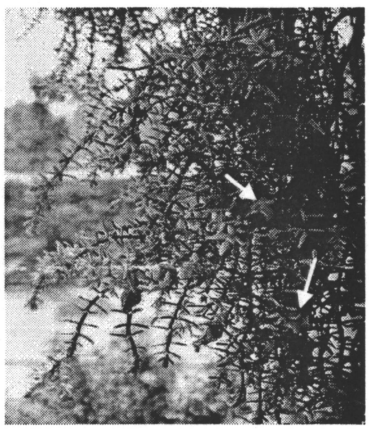

Fig. 3 - Detail of berry-like seed cones (arrows), orange (immature) in a female plant (left). The male plant has male cones and empty female like cones (right). The leaves are acuminated with two stomatiferous bands above (adaxially).

Sterilization procedures

The explants were placed in tap water for 5 minutes. Shortly after, they were sterilized with alcohol at 70% for 2 minutes and then rinsed 3 times with bidistilled sterilized water (BSW). The explants were then submerged in 3% sodium hypochlorite for 20 minutes and rinsed again with BSW. The next treatment involved in 1% fungicide Benomil dissolved in BSW for 10 minutes followed by 3 rinses with BSW. Finally the explants were placed in 70% ethanol and hand agitated for 2 min followed by 3 rinses in BSW.

In vitro cultures (multiplication)

To test the influence of medium composition and PGRs on shoot propagation, decontaminated explants (1 cm) with at least one axillary bud were placed on different semi-solid media (Table 1). Each treatment consisted of 16 culture test tubes with 10 ml of medium.

Table 1- Growth regulator combinations used in the 6 different tested media, on *Juniperus navicularis* multiplication treatments. Concentrations of PGRs are given in µM and in mg l^{-1}

PGR Combinations	Growth Regulators					
	KIN		BAP		NAA	
	µM	mg l^{-1}	µM	mg l^{-1}	µM	mg l^{-1}
Variant 1	0,93	0,20				
Variant 2	2,32	0,50				
Variant 3			0,45	0,10		
Variant 4			4,43	1,00		
Variant 5			0,90	0,20	2,74	0,51
Variant 6	0,93	0,20	0,45	0,10	2,74	0,51

Used media: MS: Murashige e Skoog, 1962; WPM: Lloyd & McCown, 1981; GD: Gupta e Durzan, 1985; SH: Shenk & Hildebrandt, 1972; AE: Arnold & Eriksson, 1981; WH: White, 1942

After one month, different parameters were evaluated, in this first multiplication cycle: nº of lateral buds developed; number of basal buds developed; length of the longest shoots (including the length of longest lateral shoot when the principal shoot was cut); colour of the basal leaves in contact with the medium; general colour of the explant; general aspect of the buds (vitrified; abnormal; normal, etc). To all the parameters a numerical scale (from 0 to 5; 5 being the best condition) was applied for use in the

statistical analysis. Table 2 summarises the parameters evaluated and the numerical scale applied for statistical analysis of the results.

Table 2: Parameters used to standardize values for further statistical analysis.

	Attributed scale number					
	0	1	2	3	4	5
Number of lateral buds	0	1		2		>2
Buds development	---	Little		Developed		Good Growth
Principal shoot	dead					alive
Length of the longest shoot		< M - M×0,2		= M ± M×0,2		> M + M×0,2
Colour of the explant		Brown	yellow	green/ brown	green/ yellow	green
Colour of the basal leaves		Brown		yellow		green
N° of buds developed at the base of the explant	0	1		2		>2
Appearance of the base buds	---	Vitrified		Abnormal develop.		normal

M: Average growth

Multiplication cycle took approximately 28 days, with culture conditions of 16h/8h light and darkness period, at 24°C/19°C respectively. Philips Master LD36W/840 Cool White lamps were used at an intensity of 90 $\mu mol\ m^{-2}\ s^{-1}$.

2nd and 3rd multiplication cycles

The influence of culture cycle and media on shoot multiplication was evaluated by comparing shoots response in different culture periods: 1st culture (shoots derived from the field mother plant), 2nd culture (shoots derived from 1-month old shoots of the 1st culture) and so on. Number of shoots per explant was evaluated in each culture period. Shoots obtained in first phase were separated from the original explants and were transferred to flasks with the new medium. In accordance with the results obtained in the first multiplication cycle, two media were selected to perform more tests (MS Variant 1 and GD Variant 3). The effect of two different concentrations of Phytagel ($3g\ l^{-1}$ and $6g\ l^{-1}$) and plants sex variations in the multiplication rates were tested. A new evaluation was performed after one month in the culture medium.

A third phase of multiplication was done in order to compare the most efficient basal medium/PGR association in the two earlier phases (GD33; GD36; MS33 and MS36;

all media supplemented with 0,45 μM BAP and with either 3 or 6 g l^{-1} of gellan gum/Phytagel, Sigma) in comparison with Olive medium (OM) (Rugini, 1984) usually used in micropropagation of other Juniper species. OM medium also contained 0,45 μM BAP (OM33 and OM36 with 3 or 6 g l^{-1} of gellan gum respectively).

In both multiplication cycles, culture conditions were the same used in the first multiplication cycle.

Rooting treatment with NAA

Fifty shoots developed during multiplication experiments were placed in GD medium (Gupta and Durzan, 1985) with (2 g l^{-1}) activated charcoal (GD0C), and left for one week under 16/8 h photoperiod and 24/19 °C temperatures, respectively. Plantlets were cleared from any browning tissues and transferred to half macronutrients WPM medium with 5,35μM (1mg l^{-1}) of naphthaleneacetic acid (NAA) where they remained for two weeks. Culture conditions were 19°C at total darkness for one week and 19°C with photoperiod of 16/8 h day/night for another week. After the induction treatment the plantlets were transferred to expression medium half WPM without PGRs. Plantlets remained in this medium for approximately six weeks, under 16/8 h photoperiod and 24/19°C day/night temperatures. Philips Master LD36W/840 Cool White lamps were used at an intensity of 90 μmol m^{-2} s^{-1}. They were periodically observed for new roots.

Rooting treatment with IBA

As in the previous treatment, the plantlets were placed in GD0C (Gupta and Durzan, 1985) medium for or one week. IBA at 3000 ppm was prepared and sterilized so plantlets could be dipped in for 5 seconds in aseptic conditions under laminar flow cabinet and immediately transferred to modified WPM root expression medium. Culture conditions were similar as for the NAA treatment, also remaining in the culture chamber for 6 weeks. During that time, plantlets were periodically observed for new roots.

Another treatment was carried out comparing IBA at 3000 ppm with IBA at 8000 ppm. Plantlets were previously cultured for one week in GD0C, with activated charcoal (as before). Afterwards, they were stripped of leaves for about 5 mm from the base and several little cuts were made in that area so the PGRse could penetrate the inner tissues. Half of the treated shoots were dipped in IBA 3000 ppm and the other half in IBA 8000

ppm for 5 seconds and placed in WPM expression medium for 6 weeks. The culture conditions were 16/8h photoperiod with 24°C/19°C of day/night temperatures. The light intensity was the same as in the other experiments previously described. The plantlets were frequently observed for new roots.

Analysis of the results

Different approaches to analyse the collected data were used. Statistical analysis of the results was made using factorial or two ways *ANOVA* analysis for mean comparison between media x PGR combination and the parameters under study. All statistical studies were performed using Statistica vs. 8 Stat Soft Inc.

RESULTS AND DISCUSSION

Multiplication (1st cycle)

In the first multiplication experiments there was several contaminations of the cultures. Since the plant material came from wild populations, this was expected. It basically demonstrated that, despite all the aseptic precautions and sterilization procedures, working with field materials has its setbacks. Fortunately, the use of single explants culture vessels allowed restriction of infected explants contamination.

After two weeks of culture, the explants on three culture media started to show general yellowing/browning, most likely due to inappropriate combination of nutrients for this species. After 3 weeks, cultures on AE, SH and HW media were discarded due to the mentioned response of the explants. As a result they have not been analysed statistically.

Explants on the other three media (MS, WPM and GD) developed lateral shoots, efficiently multiplying the explants (Figs. 4 to 7).

On Fig. 4 (left), results of the effect of medium and PGR on the number of new lateral buds is shown. The medium composition was the prime responsible for the variations on the results, being GD (nº 3 in the graphics) statistically different from the MS (1) and WPM (2) when used combined with 0,45 μM BAP (PGR 3), 0,90μM BAP (PGR 5) and 0,93 μM KIN + 0,45 μM BAP + 2,74μM NAA. There were no differences between these PGR combinations for the GD medium, what means that in all of them 2 or more new buds were formed. In all WPM x PGR combinations the number of new buds

formed was lowest than in the other two media. In fact, GD medium produced more buds that any other medium studied.

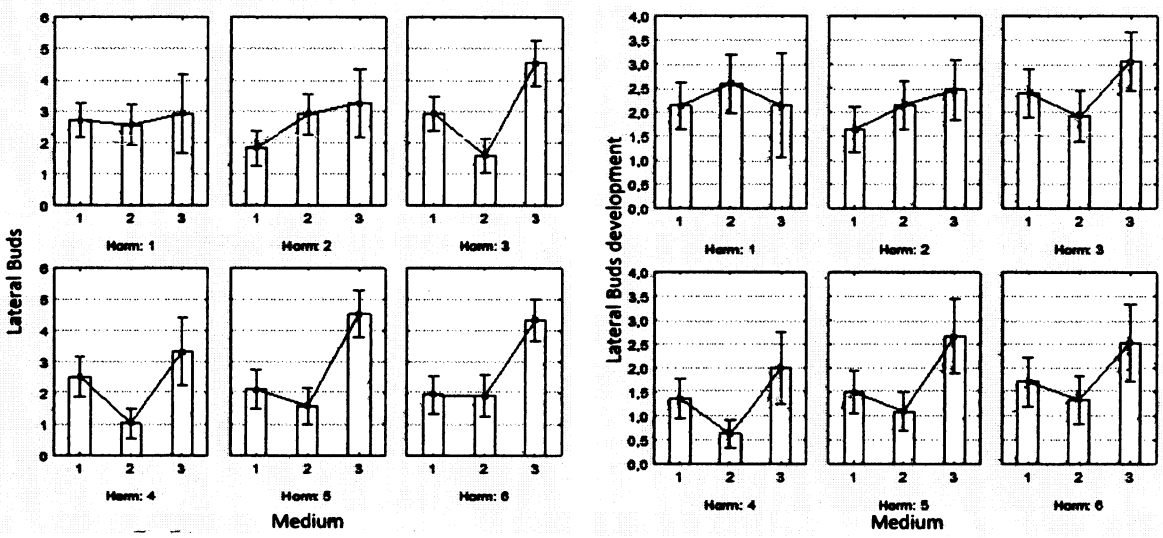

Fig. 4 - Mean plot of number of lateral buds formed (right) and of lateral buds development grouped by medium; categorized by PGR (See table 1 for media x PGR combinations). Vertical bars denote 0,95 confidence intervals.

For the lateral buds development parameter (Fig. 4 right), KIN and BAP shows independently of the medium better results (PGR 1, 2, 3). The addition of NAA or high concentrations of PGRs was negative correlated with buds developments. Considering the parameter analysed before, GD combined with 0,45 μM BAP not only formed more buds but also their development were higher than any other combination.

Main shoot status analysis can be seen in Fig. 5 (left). The results were more similar than the observed for lateral the number of new buds parameter. When KIN was used no significant differences was observed between the media tested. On the contrary when BAP was applied at 0,45 μM (PGR 4) or at 0,90μM, significant differences were observed between all media used. GD maintained more main shoots alive that any other medium, being again the best medium, for almost all PGR type and concentration used with it. WPM medium show the poorest results for most PGRs. Most of the main shoots used in WPM medium were dead after the one month experimental period.

Longest shoot length (Fig. 5 right) variation between PGRs is, as expected, barely inexistent. It actually remains similar for most media used. Once more, WPM medium had negative effect.

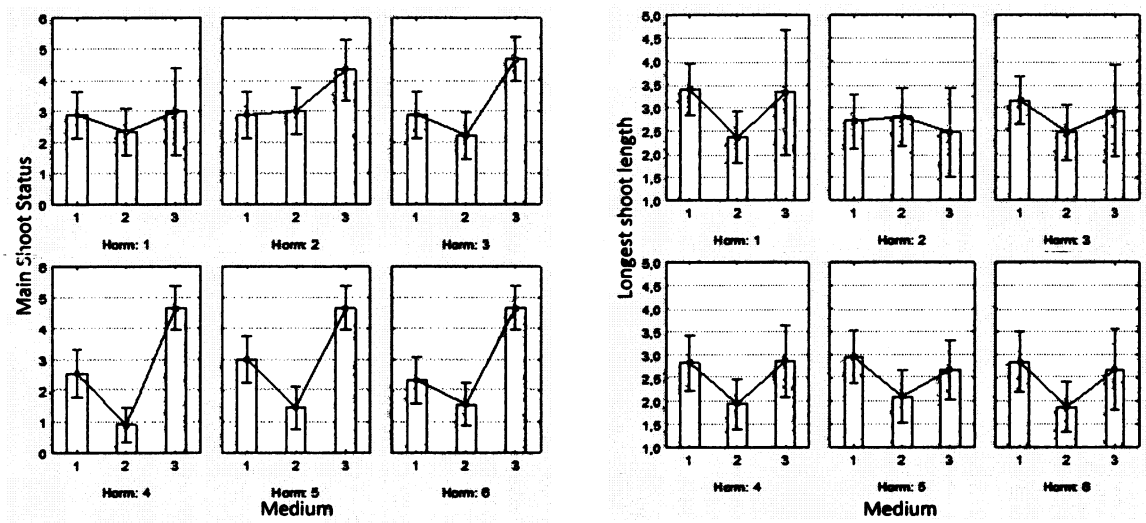

Fig. 5 - Mean plot of main shoot status (left) and longest shoot length (right) by medium; categorized by PGR. Vertical bars denote 0,95 confidence intervals.

The colour of the explant was evaluated because this parameter shows the general physiological status of the plant, and also because the media x PGR combination could have toxic effects or nutritional unbalances. There were several visible differences in the explant colour parameter (Fig. 6, left; see also Fig. 8). First of all, there is an evident difference between media. In general MS and GD medium explants maintained the natural green colour, rarely yellowing or browning. In other hand, WPM explants were normally yellowish, except for PGR 2 where this proportion was smaller. When BAP was added at a concentration higher than 0,45 μM the colour of the explants in media MS and WPM turned yellow or brown, a clear indication of toxic effects. It's also interesting that shoots of all media maintained its natural green tone when KIN was used at higher concentration.

Almost all media and PGR combination affect the colour of the basal leaves (the leaves become yellow, brown or died). Nevertheless two media x PGR combinations did not show negative effects, the leaves remained green, and in good condition. As seen in the graphics, GD3 and GD4 were quite better than all other combinations (Fig. 6, right).

One important parameter under analysis was the number of buds developed at the base of the shoots in culture (Fig. 7 left) and the appearance of the basal buds. In fact, most of the observed differences for the several PGR/medium combinations are exclusively medium related, as can be seen in the graphics. WPM medium shows the weakest results, differing significantly from the MS medium, the best for this parameter,

in PGR 1, 3 and 4. In laboratory, these results were expressed as an almost absence of basal buds in the WPM explants and as an omnipresence in MS medium explants. GD stands as an average medium for basal bud production.

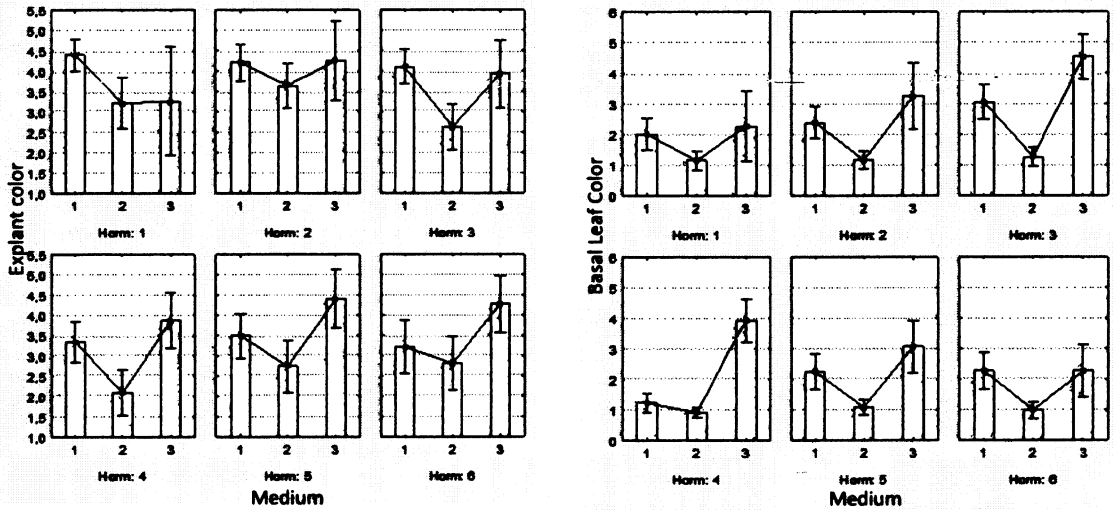

Fig. 6 - Mean plot of explant color (left) and basal leaf color (right) by medium; categorized by PGR. Vertical bars denote 0,95 confidence intervals.

The last parameter evaluated was the appearance of the basal buds (Fig. 7, right). In this specific case, KIN had less detrimental effect than all the other PGRs tested. Some of the alterations observed were vitrification, abnormal development, callus formation, and basal buds death (Fig. 8). Both PGR and medium had significant influence in the results. Independently of the basal medium used, BAP at concentration higher than 0,45 μM caused abnormal basal buds development. Buds developed under GD medium usually had better appearance than the others.

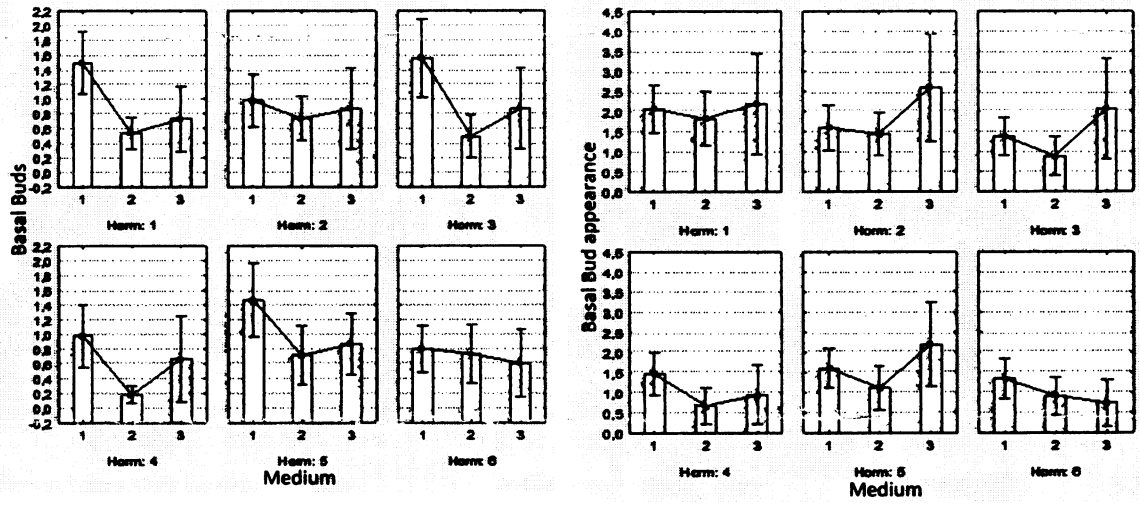

Fig. 7 Mean plot of basal buds (left) and they're appearance by medium; categorized by PGR. Vertical bars denote 0,95 confidence intervals.

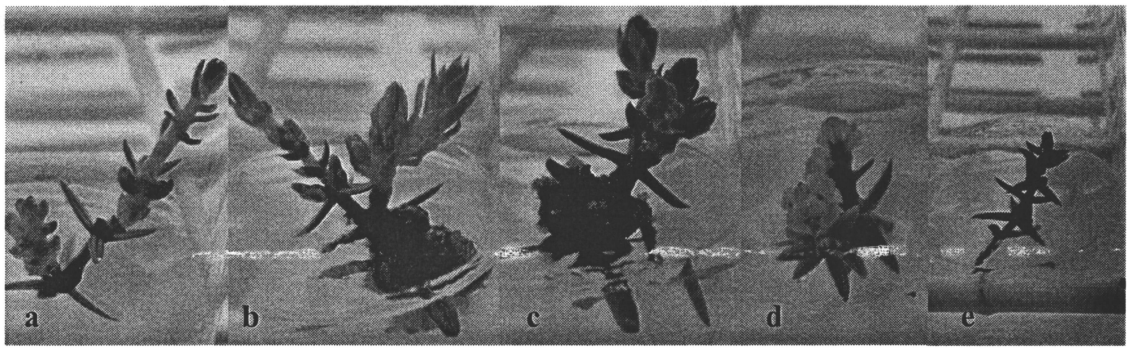

Fig. 8 - Different abnormal effects observed due to the PGR x medium combination (**a:** vitrification of the basal buds and long internodes; **b:** vitrification of the basal buds and different length of the leaves, **c:** calli development at the base of the explant; **d:** yellowish colour of the explant; **e:** death of the explant by toxic effect).

Second Multiplication Cycle

Shoots developed during the first cycle (from mother plant) were separated from the explants and used in a second cycle of multiplication. The better media x PGR combination (MS1 and GD3) was selected from results obtained in the first multiplication cycle. The objective of the new cycle was to evaluate the multiplication rate of *Juniperus navicularis*. Two different concentrations of Phytagel were used (3 and 6 g l^{-1}).

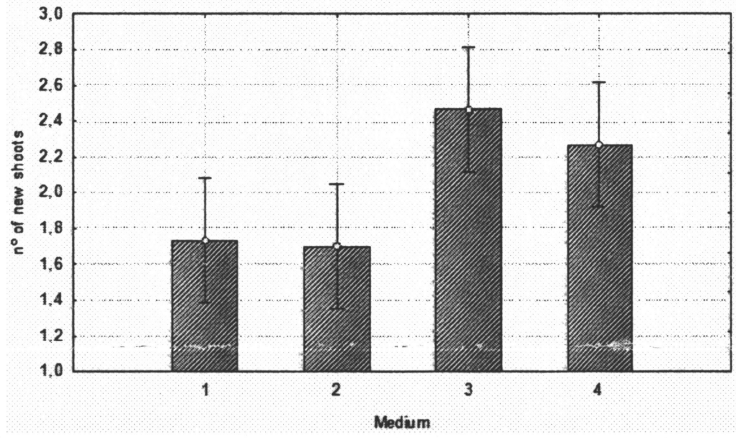

Fig. 9 - Average number of new shoots by medium tested in the second multiplication cycle. **1:** MS13 with 3 g.l^{-1} phytagel; **2:** MS16 with 6g.l^{-1} phytagel; **3:** GD33 with 3g.l^{-1} phytagel; **4:** GD36 with 6g.l^{-1} phytagel.

Through the analysis of Fig. 9, it is possible to see that GD medium with 0,45µM BAP (GD3) produced more buds than MS medium (approximately 2,45 per explant). GD produced higher amount of new buds per explant with better general aspect, although MS explants were bigger in size (data not showed). The influence of a higher or lower concentration of gelling agent was not determinant in the results obtained, anyway, in an

overall observation; a higher concentration of Phytagel slightly reduces the multiplication efficiency of the media but reduce some of the abnormal appearance of the basal buds.

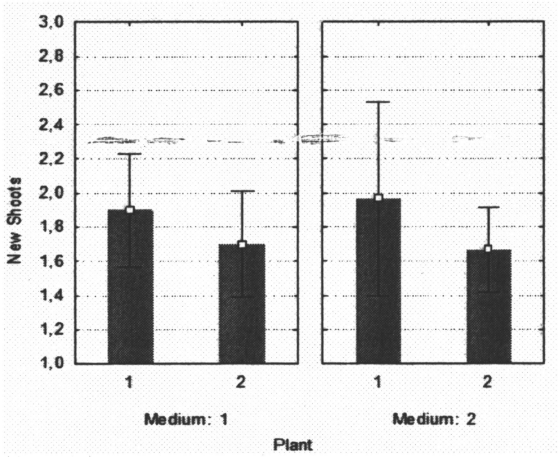

Fig. 10 - Mean plots of new shoots grouped by plant; categorized by medium. 1: Female; 2: Male; Medium 1: 3 $g.l^{-1}$ g; Medium 2: 6 $g.l^{-1}$ g.

Fig. 10 resumes the influence of the explant sex in the multiplication rates. Although there are no statistical differences in the multiplication efficiency of male and male explants, female plants had a slightly higher number of new buds, but when 6 g l^{-1} of Phytagel was used a higher variation within female plants was observed for this character. It was observed that female plantlets grew greener, with smaller leaves and higher amount of lateral buds than male plantlets. This may be because male plants were more lignified and with longer leaves.

Third Multiplication Cycle

In the third phase of multiplication OM medium was tested against the most efficient basal medium/PGR associations in the previous multiplication cycles (GD33; GD36; MS33 and MS36). The results could be seen in Fig. 11. In general, higher number of new shoots was observed for almost all media tested comparatively with first and second cycle (compare with Fig. 9 and 10). This fact could be explained by a reduction in the level of stress imposed by the activated charcoal elongation process; on the other hand, shoots need to adjust to the *in vitro* conditions, as they derived from stems of field plants, and cultures go through an adaptation period to *in vitro* culture. These results are in agreement with Loureiro *et al.*(2007) for *Juniperus phoenicea.*

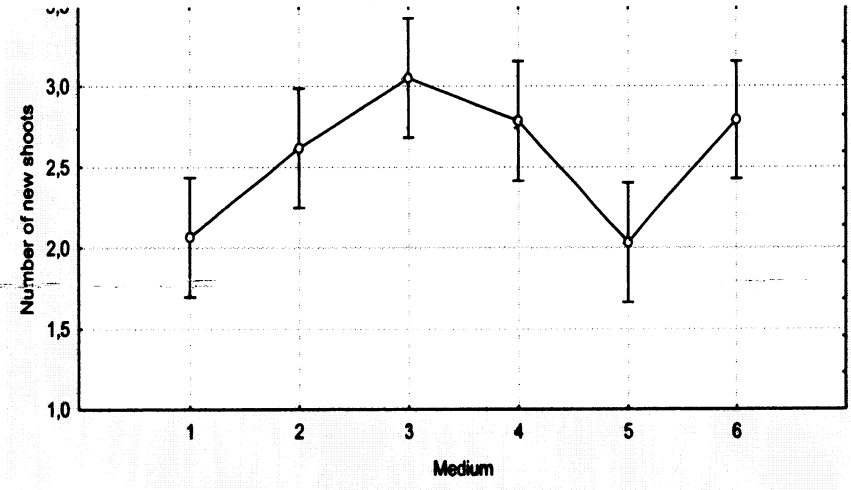

Fig. 11 - Effect of the culture medium on the number of new shoots formed per shoot after one month in culture during the third cycle of multiplication. Current effect F(5,365) =4,9276; p=0,00022. Vertical bars denote 0,95 confident intervals. Media: **1:** GD33; **2:** GD36; **3:** OM33; **4:** OM36; **5:** MS33; **6:** MS36.

There were no significant differences between GD36; OM33; OM36 and MS36, even though OM33 (supplemented with 3 mg l^{-1} of Phytagel) produced the highest number of new shoots (an average of 3,06 new shoots). Other important observation was the fact that on the second cycle, media supplemented with 6 mg l^{-1} of Phytagel produced less number of new shoots. In the case of the third cycle, GD36 and MS36 were superior to the same media but supplemented with 3 mg l^{-1} of Phytagel (compare Figs. 9 and 11).

Rooting treatment with NAA

Rooting experiment made using NAA was unsuccessful, with none of the treated plantlets producing roots after 6 weeks. On the other hand, great aerial part development was recorded, with some of the plantlets growing almost 8 times the original size (from 1 cm to 8 cm tall). Lateral buds were active but had minor development (2 mm). Larger plantlets showed 4 to 6 of these buds. Major formation of callus, from which new buds appeared, was also visible. These new buds however, were vitrified, therefore inappropriate for *in vitro* cultures.

Rooting treatment with IBA

None of the rooting treatments with IBA showed root formation after 6 weeks. Nonetheless, extensive callus formation could be seen in plants treated with 3000 ppm IBA. Plantlets treated with higher concentrations of IBA tend to show browning in the leaves and lower growth, possibly a physiological response to the excess of PGR.

Rooting difficulties are known in conifers either from cuttings or microshoots and several research reports have been published in this area in order to improve root establishment (Hamann, 1998; Oliveira, 2003, Ragonezi, 2008).

CONCLUSIONS

Juniperus navicularis responded well to various multiplication media i.e., OM; GD and, in some cases, MS (in this order) if BAP (0.45 μM) and 3 or 6 mg l^{-1} of gellan gum was used. Nevertheless, further investigation must be done in relation to the gelling agent, because contradictory results were observed between the second and third multiplication media. The medium used in combination with the specific PGR and its concentration affected drastically the results obtained. The general status (health) of the shoots in culture, (evaluated through different qualitative parameters as the colour of the explant, incidence of basal shoots vitrification, morphological abnormalities) showed that GD medium had the best results with almost all PGR used. BAP was the best choice for almost all basal media tested, but only when concentrations were not higher than 0.45 μM. With this shoot multiplication protocol, it is possible to obtain more 500 explants from some initial 20, after 3 multiplication cycles.

In spite of the establishment of the shoot multiplication protocol, no rooting was achieved in *in vitro* experiments. The obvious conclusions of the rooting experiments are that *J. navicularis* did not seem to react neither to NAA or IBA treatments, at least to the concentrations tested.

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Annex

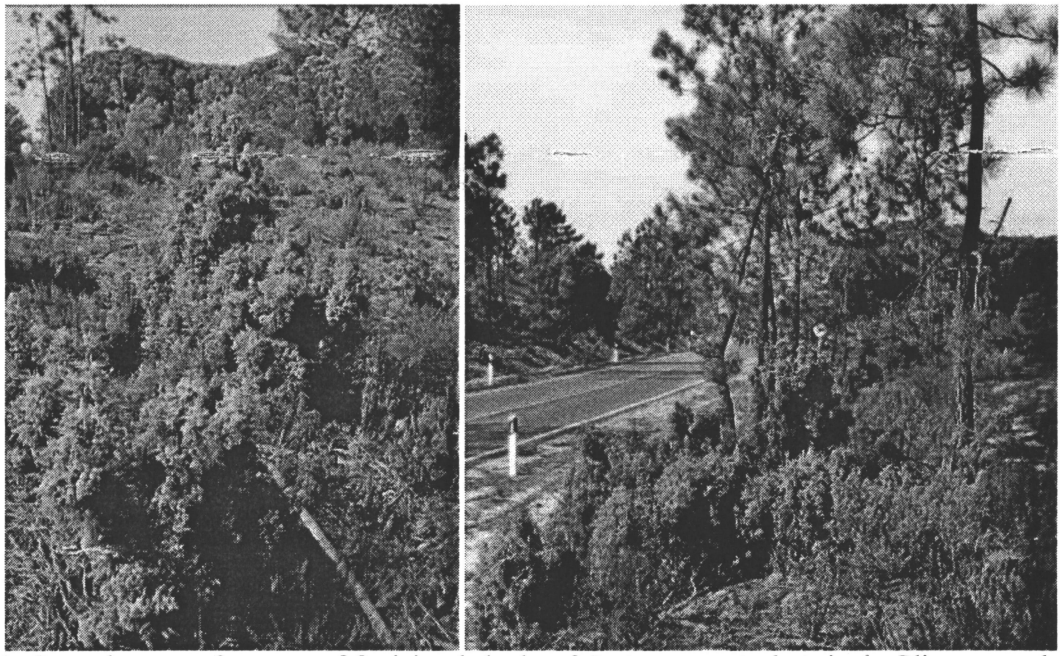

Fig. 1: General aspect of a group of fastigiated shrubs of *Juniperus navicularis* in the Pliocene sands near the sea coast of Portugal (left). A group of *Juniperus navicularis* between Melides and Alcácer do Sal.

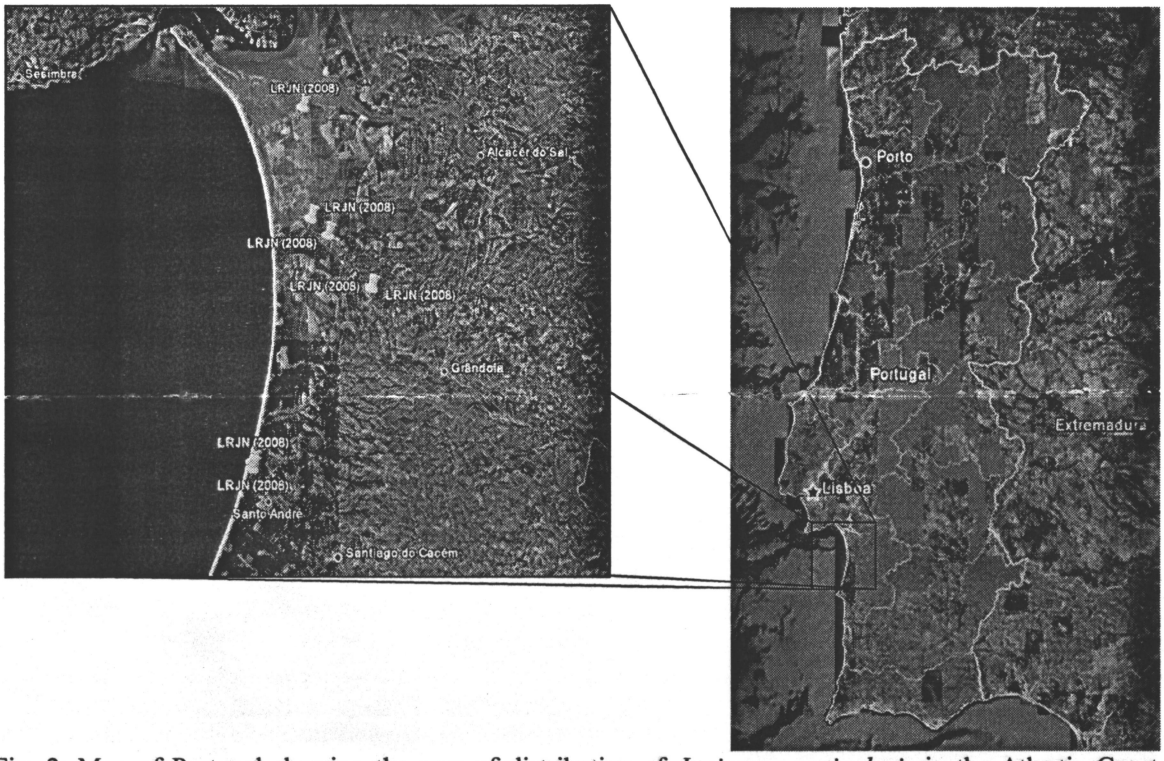

Fig. 2: Map of Portugal showing the area of distribution of *Juniperus navicularis* in the Atlantic Coast (right) and specific location where plant material was collected for in vitro cultures studies (left).

CONSIDERAÇÕES FINAIS

Grande parte das populações de *J. navicularis* que haviam sido documentadas anteriormente foi extinta ou encontra-se em risco de destruição eminente devido a actividades antropogénicas. As que ainda persistem, demonstram diferentes tipos de respostas de acordo com o tipo de manutenção a que a área da sua ocorrência é submetida. Enquanto populações pouco perturbadas tende a distribuir-se aleatoriamente, sem diferenças em termos de rácio macho/fêmea, as populações que se encontram em recuperação após arroteia ou corte tendem a ser mais agregadas, com uma maior discrepância sexual. Ao contrário dos resultados obtidos, a proporção de plantas maduras e imaturas deveria ser mais equilibrada para todas as populações.

O facto dos juniperos, assim como outras coníferas, apresentam severas debilidades em termos de germinação das sementes, implica que grande parte dos indivíduos das populações surja por reprodução vegetativa, o que reduz drasticamente a variabilidade genética da espécie no local.

Baseado nestes factos, acções de conservação devem ser tomadas de forma a salvaguardas esta espécie única. A troca de plantas entre populações com mesma base genética, de forma a melhorar a variabilidade genética das mesmas, assim como limitar os cortes e arroteias efectuados em áreas de ocorrência podem ajudar as populações existentes a recuperar.

Como alternativa, o recurso a técnicas de cultura *in vitro* é uma hipótese cada vez mais utilizada para a conservação de espécies em perigo, nomeadamente para a manutenção das características próprias de cada população. A germinação *ex-situ* e a cultura de tecidos são algumas das alternativas à conservação *in situ*, o que me levou a considerar a segunda parte deste trabalho.

O *J. navicularis* respondeu bem aos vários meios de multiplicação. No entanto, foram visíveis diferentes respostas para cada combinação meio/regulador de crescimento, afectando drasticamente os resultados obtidos. O regulador de crescimento BAP demonstrou os melhores resultados quando utilizado em baixas concentrações nos meios. Com o protocolo de multiplicação definido neste trabalho, é possível, a partir de 20 explants, obter mais de 500 plantulas, após 3 ciclos de multiplicação que demoraram pouco mais de 80 dias. Apesar do estabelecimento deste protocolo de multiplicação de rebentos, não foram obtidos resultados em nenhum dos ensaios de enraizamento *in vitro*. Estas dificuldades são conhecidas em coníferas, tanto para estacas como para

rebentos *in vitro*, e vários trabalhos foram já publicados na área, de forma a melhorar o aparecimento e desenvolvimento de raízes (Hamann, 1998; Oliveira et al., 2003, Ragonezi et al., 2008).

Contudo, novos ensaios devem ser realizados para estudar os agentes de gelificação, dado que algumas variações foram verificadas consoante as concentrações utilizadas nos meios. Ensaios de enraizamento devem ser também preparados para estabelecer totalmente o protocolo de multiplicação, sendo assim possível preparar as plantas para aclimatação e posteriormente para a recuperação de populações *in situ*.

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