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RELATIONSHIPS BETWEEN MATURE CONE TRAITS AND SEED VIABILITY IN *JUNIPERUS OXYCEDRUS* L. SUBSP. *MACROCARPA* (SM.) BALL (CUPRESSACEAE)

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The study addressed different biological aspects of *Juniperus oxycedrus* subsp. *macrocarpa*, including female cone production, biometric analysis of mature cones and seeds, and their viability. The results indicate that the proportion of aborted female cones was very high. In the four populations studied, cone diameter and weight and seed length and weight were closely related. The distribution of numbers of seeds with and without embryos in mature cones was examined, as well as the frequency of numbers of seeds with embryos. The general trend in all populations was three seeds per mature cone, of which only one or two showed embryos. Tetrazolium tests showed the percentage of viable seeds to be lower.

Key words: Biometric, fertility, tetrazolium test, *Juniperus*, Cupressaceae.

INTRODUCTION

Using material from Greece, Smith (1816) described coastal juniper and named it *Juniperus macrocarpa*. Later, Ball (1878) classified it as a subspecies of *Juniperus oxycedrus* L., a taxonomic status still supported by different authors (Amaral Franco, 1986, 1993; Fernández Galiano, 1987). Recent studies based on leaf essential oils and molecular DNA data (Adams, 2000) point to its delimitation as a species. This taxon has a relatively wide distribution in the Mediterranean region (Coode and Cullen, 1965; Jalas and Suominen, 1973; Boratynski and Browicz, 1982; Amaral Franco, 1986, 1993), although nowadays in the Iberian Peninsula its population is distributed in a discontinuous pattern along a narrow coastal strip (Pastor and Juan, 1999). This fragmentation, due to the high human pressure exerted on the populations, has caused it to be considered an endangered species (Blanca et al., 1999) and subject to a recovery program.

Among the gymnosperms, the genus *Juniperus* is one of the few that develops fleshy mature cones and, according to Herrera (1992), has functional behavior similar to angiosperms with fleshy fruits. We studied different biological aspects of *J. oxycedrus* subsp. *macrocarpa*, including female cone production, biometric analysis of mature cones and seeds, and their viability, because differences between these characters at the population level have been found in other *Juniperus* species such as *J. communis* var. *depressa* (Houle and Babeux, 1994).

Cone production, exhibiting very significant interannual fluctuations, is one of the most common problems in conifers. According to Houle and Filion (1993) or García et al. (2002), this variability in production is due in most cases to climatic factors. On the other hand, Ortiz et al. (1998) cite the pollination period as the most critical factor in *Juniperus oxycedrus* because the bulk of seed and cone abortion occurs during this stage, indicating that pollen is essential for seed strobilus development.

Generally, biometric studies in the genus *Juniperus* have been very useful taxonomically, allowing interspecific and infraspecific differences

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TABLE 1. Characteristics of four populations of *Juniperus oxycedrus* subsp. *macrocarpa* in the SW littoral of Spain

Populations	Status	Altitude (m)	Geographic location	Habitat
Punta Umbría (PU)	Natural site	0	37° 13' N – 6° 57' W	Dunes and pine forest
Doñana (DO)	National park	0–10	36° 48' N – 6° 22' W	Dunes
Caños de Meca (CM)	Natural park	100	36° 11' N – 5° 55' W	Pine forest and sea cliff
Punta Paloma (PP)	Unprotected site	0	36° 00' N – 5° 43' W	Dunes and pine forest

to be established (Smith, 1816; Ball, 1878; Lebreton and Thivend, 1981; Gauquelin et al., 1988; Lebreton and Rivera, 1988; Lebreton et al., 1991). However, attention recently has turned to the relation between these aspects, basically seed size and adaptation capacity (Hewitt, 1998), germination processes (Dunlops and Barnett, 1983; Vera, 1997) and seedling development and survival (Jurado and Westoby, 1992; Ocaña et al., 1997; García et al., 2002).

We assessed viability because most species of *Juniperus* show difficulties in the process of seed germination. It is also well known that one of the principal ecological problems in Cupressaceae is low production of viable seed (Colangeli and Owens, 1990; Owens et al., 1990; Owens and Schliesing, 1995; Ortiz et al., 1998).

At present this taxon is clearly in regression in the coastal zones of southwestern Spain, in part due to anthropogenic disturbances.

The aim of this study is to examine the reproductive behavior of this species: (1) to study the biometric characters of mature cones and seeds; (2) to search for a successful method of selecting mature cones and seeds; (3) to analyze the percentages of seeds with or without embryos in order to observe any pattern; and (4) to verify the viability of seeds with embryos by means of the tetrazolium test.

MATERIALS AND METHODS

Juniperus oxycedrus subsp. *macrocarpa* is a dioecious plant, generally a shrub in the studied area. Its vegetative growth occurs basically during spring and to a lesser extent in autumn. Flowering occurs from October to December and the cones mature and become fleshy in the second year, between September and December.

This study was carried out from 1997 to 1999 in four different populations in the SW littoral of Spain (Tab. 1).

To estimate fertility rates, ten individuals in each population (PU, DO, CM, PP) were randomly chosen, and in each one five branches were marked and its female cones were counted. Afterwards the number of these cones that ripened were also counted.

Fresh mature cones were examined in each population, over a sample of 200–400 obtained from ten individuals. Diameter and weight were measured and the number of included seeds was counted.

Biometric data on length and weight were obtained from 3925 seeds taken from mature cones, considering the four populations.

To assess seed viability, the cutting method was applied in the first instance: each seed was opened to determine the presence or absence of an embryo. This method cannot distinguish embryos that look normal but are not viable, so the topographic tetrazolium method was employed: the seeds were immersed in 2,3,5-triphenyltetrazolium chloride solution as indicated by international rules methodology (ISTA, 1976, 1999).

Finally, to establish the relationship between seed viability and sinking/floating mature cones and sinking/floating seeds, a sample consisting of 20,000 mature cones taken from the four populations was used. The mature cones were kept in water for 48 h, the sinking and floating ones were separated, and seeds were extracted. Seeds from sinking mature cones and from floating ones were separated by floatability.

The data were analyzed by one-way or two-way ANOVA and, using Tukey's test where the differences were significant, the means were separated. Prior to statistical analyses, variables were checked for normality and transformed when necessary (Steele and Torries, 1980).

RESULTS

FERTILITY

The mean percentage of cone formation varied from 8.39% in Punta Umbría to 12.10% in Doñana

TABLE 2. Summary of several cone traits in four *Juniperus oxycedrus* subsp. *macrocarpa* populations. Different letters in same column indicate significant differences between values (Tukey's test, $p < 0.001$)

Population	Mature cone production (%)			Weight and size of cones				
	mean ± se	Range	n	weight (g) (mean ± se)	range	diameter (mm) (mean ± se)	range	n
Punta Umbría	8.39 ± 3.56[a]	4.65–15.55	10	1.94 ± 0.75[a]	0.72–4.94	16.37 ± 2.16[a]	11.13–23.8	400
Doñana	12.10 ± 6.77[a]	1.25– 9.20	10	1.36 ± 0.47[b]	0.47–4.23	15.40 ± 1.85[b]	10.80–21.5	400
Caños de Meca	10.23 ± 8.91[a]	2.25–33.08	10	1.50 ± 0.52[c]	0.52–3.68	15.23 ± 1.57[b]	11.30–20.0	400
Punta Paloma	11.16 ± 3.78[a]	5.73–17.30	10	1.75 ± 0.57[a]	0.85–3.48	15.23 ± 1.56[b]	12.40–18.8	200

TABLE 3. Summary of seed weight and length in four *Juniperus oxycedrus* subsp. *macrocarpa* populations. Different letters in same column indicate significant differences between values (Tukey's test, $p < 0.001$)

Population	Seeds				n
	weight (g) (mean ± se)	range	length (mm) (mean ± se)	range	
Punta Umbría	0.08 ± 0.03[a]	0.01–0.20	7.20 ± 0.90[a]	3.95–9.7	1150
Doñana	0.09 ± 0.03[b]	0.01–0.30	7.66 ± 0.83[b]	4.20–9.9	1200
Caños de Meca	0.09 ± 0.03[b]	0.01–0.21	7.61 ± 0.86[b]	4.20–9.6	1050
Punta Paloma	0.11 ± 0.04[c]	0.02–0.26	7.54 ± 0.92[b]	4.90–9.7	625

TABLE 4. Pearson's product-moment correlation coefficients (cc) between cone weight and diameter, and between seed weight and length in each *Juniperus oxycedrus* subsp. *macrocarpa* population studied

Population	Cone weight – cone diameter		Seed weight – seed length	
	cc	n	cc	n
Punta Umbría	0.8659*	400	0.5577*	1150
Doñana	0.8744*	400	0.6592*	1200
Caños de Meca	0.7989*	400	0.6610*	1050
Punta Paloma	0.8861*	200	0.5576*	625

* $p < 0.05$

(Tab. 2), without significant differences among the four studied populations. Despite the similar behavior observed in the four populations examined, there was great intrapopulation variability, with Caños de Meca being the population with the highest range of variation (2.25–33.08) (Tab. 2).

MATURE CONES AND SEEDS STUDY

Mature cone weight varied from 1.36 g in Doñana to 1.94 g in Punta Umbría (Tab. 2). There were three significantly different groups ($F = 71.27$, df 3–1396, $p < 0.001$): the first formed by the populations of Punta Umbría and Punta Paloma, the second formed by the Caños de Meca population, and the third by the Doñana population. Regarding size, mature cones were clearly bigger in Punta Umbría ($F = 29.85$, df 3–1396, $p < 0.001$) (Tab. 2). Mature cone weight and size correlated well in the four populations, especially in Punta Paloma ($r = 0.886$) and Doñana ($r = 0.874$) (Tab. 4).

Seed weight varied from 0.08 g in Punta Umbría to 0.11 g in Punta Paloma (Tab. 3). Three different groups were distinguished here as well ($F = 46.67$, df 3–4021, $p < 0.001$), the first being Punta Umbría, the second including Doñana and Caños de Meca, and the third Punta Paloma. Seed size presented higher homogeneity; only the Punta Umbría seeds, being smaller, showed differences from those of the other populations ($F = 63.18$, df 3–4021, $p < 0.001$).

TABLE 5. Mean number of seeds per cone and frequency of cones with 1, 2, 3 or >3 seeds in each *Juniperus oxycedrus* subsp. *macrocarpa* population studied. Different letters in the same column indicate significant differences between values (Tukey's test, $p < 0.001$)

Population	No. of seeds/ cone		% of cones with n seeds (mean ± se)				n
	mean ± se	range	1s	2s	3s	>3s	
Punta Umbría	2.89 ± 0.91^a	1–8	4.68 ± 6.06	28.61 ± 14.60	57.33 ± 14.51*	9.38 ± 15.20	400
Doñana	2.96 ± 0.79^a	1–7	3.02 ± 2.58	16.38 ± 12.27	68.28 ± 11.44**	12.07 ± 7.14	400
Caños de Meca	2.52 ± 0.72^b	1–6	9.05 ± 7.67	31.35 ± 9.57	56.14 ± 10.19*	3.46 ± 6.32	400
Punta Paloma	2.46 ± 0.77^b	1–5	14.00 ± 21.83	29.00 ± 11.97	55.00 ± 20.95*	2.00 ± 2.58	200

* $p < 0.01$, ** $p < 0.001$

TABLE 6. Pearson's product-moment correlation coefficients (cc) between number of seeds per cone and three other traits of *Juniperus oxycedrus* subsp. *macrocarpa* in the four examined populations

Population	Cone weight (g)		No. of embryos /cone		Seed weight (g)	
	cc	n	cc	n	cc	n
Punta Umbría	0.4655*	400	0.4717*	400	–0.0570	1150
Doñana	0.5217*	400	0.4643*	400	–0.0452	1200
Caños de Meca	0.4829*	400	0.4422*	400	–0.0632	1050
Punta Paloma	0.3719*	200	0.4831*	200	–0.0816	625

* $p < 0.05$

As seen in mature cones, seed weight and length correlated (Tab. 4).

Although seed number per mature cone could be quite high (up to 8 in Punta Umbría), the mean value was less than three in all populations (Tab. 5). Mature cones with three seeds predominated in the four studied populations, which always had percentages above 50% (Tab. 5). The differences between numbers of cones with three seeds and those with one, two and more than three seeds were significant ($p < 0.01$ in Punta Umbría, Caños de Meca and Punta Paloma; $p < 0.001$ in Doñana). This pattern is similar in all the populations, with a small percentage of mature cones with only one and more than three seeds, a significantly different number with two seeds in Punta Umbría ($p < 0.01$) and Caños de Meca ($p < 0.001$), and the majority of the cones having three seeds. Figure 1 represents the reproductive behavior of the four studied populations, with the state of seeds in the different kinds of mature cone specified. Usually at least one of the seeds of the cone was empty, mainly in the mature cones with two or three seeds. Because of this, and because cones with 2 and 3 seeds were the most frequent ones, the usual finding was one or two seeds with embryos per cone (Fig. 1b). The only exception was Doñana, where the higher proportion of seeds with embryos per mature cone usually coincided with the number of seeds, so that cones with two or three embryos were predominant (Fig. 1a,b). In terms of number of seeds with embryos per cone, there were three significantly different groups in the four populations ($p < 0.001$): first, cones with more than three seeds with embryos; second, those with three seeds with embryos or cones with seeds without embryos; and third, those with one or two seeds with embryos (Fig. 2).

The number of seeds per mature cone and cone weight correlated (Tab. 6). However, in none of the examined populations was there a correlation between the number of seeds and seed weight, although there was a relation between the number of seeds per mature cone and the number of embryos in each cone (Tab. 6).

VIABILITY

The frequency of seeds with embryos was checked using the cutting method; it was similar in the four populations, ranging from 55.85% in Punta Paloma to 64.81% in Doñana (Tab. 7). Later the tetrazolium test indicated that the rate of viable embryos was lower: the percentage of completely dyed seeds ranged from 6.43% in Punta Paloma to 11.92% in Doñana (Tab. 7).

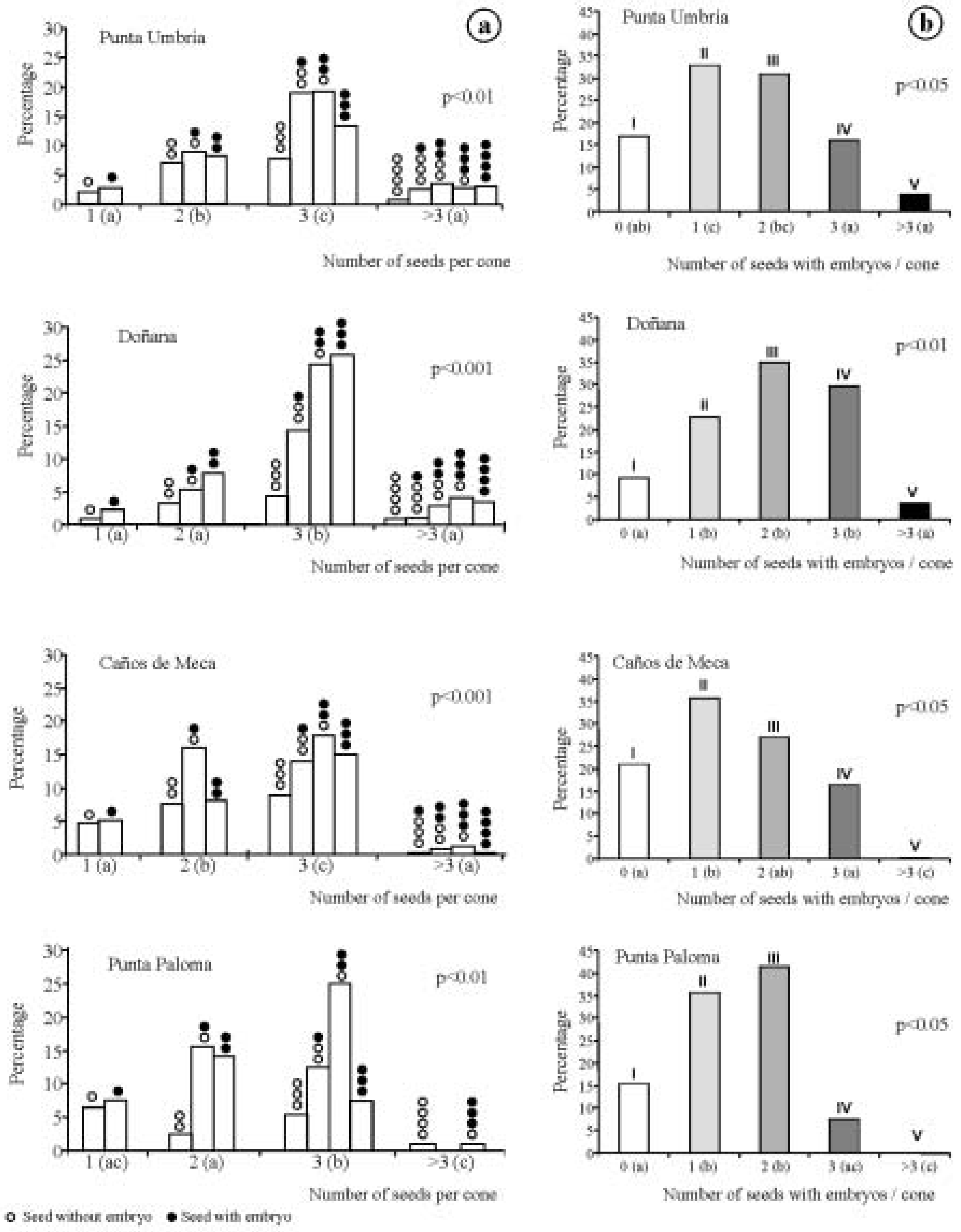

Fig. 1. (**a**) Distribution of numbers of seeds with and without embryos among mature cones in four *J. oxycedrus* subsp. *macrocarpa* populations, (**b**) Frequency of numbers of seeds with embryos, regardless of number of seeds, in four *J. oxycedrus* subsp. *macrocarpa* populations. Means with the same letter do not differ significantly (Tukey's test). I – cones with n seeds, all empty; II – cones with n seeds, with only one full seed; III – cones with n seeds, with two full seeds; IV – cones with n seeds, with three full seeds; V – cones with n seeds, with more than three full seeds.

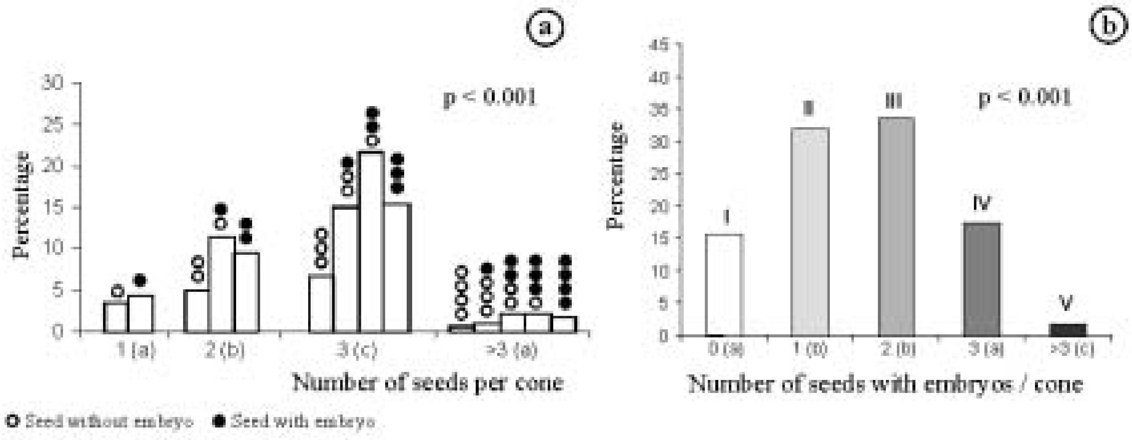

Fig. 2. (**a**) Distribution of numbers of seeds with and without embryos among the mature cones#, (**b**) Frequency of numbers of seeds with embryos, regardless of number of seeds, in all *J. oxycedrus* subsp. *macrocarpa* populations studied. Means with the same letter do not differ significantly (Turkey's test). I – cones with n seeds, all empty; II – cones with n seeds, with only one full seed; III – cones with n seeds, with two full seeds; IV – cones with n seeds, with three full seeds; V – cones with n seeds, with more than three full seeds.

METHOD FOR SEED SELECTION

Due to the high percentage of seeds without embryos in all the populations, it would be useful to find a method to eliminate as many such seeds as possible. Following Jordan de Urríes (1997) we tested the floatability of mature cones. Similar floating results were found in the four populations (Tab. 8). The percentage of floating mature cones was higher than the sinking ones in Caños de Meca and Doñana. In both kinds of mature cones, the percentage of sinking seeds did not differ significantly between the populations studied, although it ranged from 71.36% (Caños de Meca) to 84.77% (Punta Umbría) for sinking mature cones and from 58.53% (Caños de Meca) to 93.65% (Punta Paloma) for floating ones.

In samples of 1000 seeds from each population, the percentages of sinking seeds with embryos, in both sinking and floating mature cones, did not differ significantly between populations, ranging from 57% (Doñana) to 68% (Caños de Meca) in the case of sinking mature cones and also from 57% (Punta Umbría) to 68% (Doñana) in floating cones. Once all the seeds were opened, it was found that only 2.5% (Doñana) and 1% (Caños de Meca) of the floating seeds obtained from floating mature cones had embryos. Over 50% of the sinking seeds had embryos, regardless of the sort of mature cone they belonged to; this was significantly different reproductive behavior ($F = 323.65$, df 1, $p < 0.001$) (Tab. 9).

DISCUSSION

Juniperus oxycedrus subsp. *macrocarpa* presents high male and female cone production, to compensate the negative influence that dioecy combined with anemogamy (unspecific pollination) has on the amount of pollen that reaches female individuals (Jordano, 1991; Wilson et al., 1996). In some angiosperms, low reproductive success is due to low incidence of pollen and therefore low production of mature cones (Martínez-Pallé and Aronne, 2000). In any case, in all the studied populations the rate of aborted female cones or those with incomplete development was very high, over 90% in Punta Umbría. These observations differ from those of Ortiz et al. (1998), which indicate mature cone production of 19.23%, 29.39% and 38.32% for Punta Umbría, Caños de Meca and Punta Paloma (8.39%, 10.23% and 11.16% in the present study). García et al. (2001, 2002) found that cone production in *J. communis* differed significantly between years in the same population.

One of the characters typically used taxonomically is the size of ripe cones. Particularly in the genus *Juniperus* it delimits some taxa, and in *J. oxycedrus* it helps to distinguish the subspecies *oxycedrus* and *macrocarpa* (Amaral Franco, 1986, 1993; Fernández Galiano, 1987; Lebreton et al., 1991; López González, 2001). Our results show that in the population of Punta Umbría it is clearly bigger, although cone size adjusts to the recognized

TABLE 7. Summary of seed viability assessed by two methods

Population	Cutting method	Tetrazolium method			n
	% seeds with embryo (mean ± se)	% unstained	% partially stained	% totally stained	
Punta Umbría	56.53 ± 13.67	3.09 ± 5.60	44.96 ± 12.89	8.48 ± 7.00	1800
Doñana	64.81 ± 12.28	6.45 ± 8.26	46.44 ± 13.27	11.92 ± 8.11	1500
Caños de Meca	60.49 ± 16.59	3.60 ± 5.30	48.39 ± 14.99	8.50 ± 6.56	1400
Punta Paloma	55.85 ± 17.49	3.46 ± 4.00	45.96 ± 18.82	6.43 ± 5.87	1000

TABLE 8. Percentage of sinking and floating cones in samples of 5000 cones/population and frequency of sinking seeds in subsamples

Population	Sinking cones			Floating cones		
	% (mean ± se)	% sinking seeds (mean ± se)	N seeds	% (mean ± se)	% sinking seeds (mean ± se)	N seeds
Punta Umbría	60.18 ± 32.48	84.77 ± 9.34	7675	39.82 ± 32.48	84.13 ± 14.56	5211
Doñana	35.78 ± 18.82	83.90 ± 15.30	3744	64.22 ± 18.82	83.02 ± 8.44	8039
Caños de Meca	36.50 ± 15.21	71.36 ± 23.73	3690	63.50 ± 15.21	58.53 ± 16.90	6963
Punta Paloma	62.26 ± 21.04	84.26 ± 6.64	5699	37.74 ± 21.04	93.65 ± 6.31	3162

TABLE 9. Percentage of sinking and floating seeds with embryo in samples of 1000 seeds/population, in both sinking and floating cones. Different letters in the same row indicate significant difference between values (Tukey's test, $p < 0.001$). No significant differences in the same column

Population	Sinking cones		Floating cones	
	% sinking seeds with embryo (mean ± se)	% floating seeds with embryo (mean ± se)	% sinking seeds with embryo (mean ± se)	% floating seeds with embryo (mean ± se)
Punta Umbría	64 ± 12.94^a	0 ± 0^b	57 ± 19.23^a	0 ± 0^b
Doñana	57 ± 13.51^a	0 ± 0^b	68 ± 11.51^a	2.5 ± 4.33^b
Caños de Meca	68 ± 23.87^a	0 ± 0^b	67 ± 20.80^a	1.0 ± 2.24^b
Punta Paloma	65 ± 23.18^a	0 ± 0^b	63 ± 15.00^a	0 ± 0^b

range in this taxon. On the other hand, the weight of the cones shows higher variability, differing in three of the four populations studied. This may be a species characteristic, as Gauquelin et al. (1988) suggest for *J. thurifera*, where neither cone weight nor cone size were useful for separating infraspecific categories, or it may due to the conditions in which the cones developed in each population, which frequently affect seed weight (Westoby et al., 1992; Bonfil, 1998; Zhang, 1998).

Another useful biometric character in this genus is the number of seeds per cone. Previously, Gauquelin et al. (1988) separated two subspecies in *J. thurifera* according to this character and biochemical characters. In the studied populations of *J. oxycedrus* subsp. *macrocarpa*, most of the cones contained three seeds, but a trend to having more was observed in the populations of Huelva Province (Punta Umbría and Doñana). This would explain why the population of Punta Umbría had smaller seeds although it formed bigger cones. A good relation between number and weight of seeds was been found, unlike in other gymnosperms such as *J. communis* var. *depressa* (Houle and Babeux, 1994), or some angiosperms such as *Convallaria majalis* (Eriksson, 1999), *Messua ferrea* (Latif Khan et al., 1999) or *Lupinus polyphyllus* (Aniszewski et al., 2001). On the other hand, the number of seeds per cone was positively related to other characters like cone weight or the number of seeds with embryos per cone.

In the different cones there was a relatively high proportion of aborted seeds, but the percentages were much lower than those observed by Ortiz et al. (1998) in three of the four populations studied in a sample of 30 cones. The effect of population vigor on the frequency of empty seeds (Woodward, 1990; Houle and Filion, 1993) could explain why the Doñana population had a higher number of seeds with embryos; it is inside a national park so its state of conservation is better. Fuentes and Shupp (1998) suggest that in *J. osteosperma* empty seed production could be favored by natural selection as it reduces the predation rate. García et al. (2000) later observed a reduction in predation when the proportion of empty seeds increased in *J. communis*.

In our study, the general trend in all populations was for most of the cones to contain one or two seeds with embryos, in contrast to Ortiz et al. (1998) who reported most cones with empty seeds except in the Punta Paloma population, where cones with one full seed were the more frequent.

Regardless of the number of seeds per cone, the presence of aborted seeds in the genus *Juniperus* seems common, reaching up to 96% in a population of *J. communis* var. *depressa* studied by Houle and Babeux (1994). Altogether, the four examined populations of *J. oxycedrus* subsp. *macrocarpa* presented a 55–65% proportion of seeds with embryos, very similar to the 54% found by Cantos et al. (1998) in the population of Cabo Roche (Cádiz), and somewhat higher than the percentages indicated by Ortiz et al. (1998) in the populations of Punta Umbría and Barbate (Caños de Meca), which were always lower than 30%.

Compared to other subspecies of *J. oxycedrus*, subsp. *macrocarpa* is the one with the highest percentage of seeds with embryos. In subsp. *oxycedrus*, Pardos and Lázaro (1983) gave between 25% and 35%, Cantos et al. (1998) indicated 38%, and Ortiz et al. (1998) found 30.8%. In subsp. *badia*, between 24% and 48% has been cited (Jordan de Urríes, 1997). In other species the percentage is rather higher than the 4% found in *J. ashei* by Owens and Schliesing (1995).

When the tetrazolium test was applied the proportion of viable embryos decreased dramatically in all the studied populations; no case of more than 12% full seeds was found. Previously, Pardos and Lázaro (1983) reported 20% of seeds completely dyed with tetrazolium solution in subsp. *oxycedrus*; Jordan de Urríes (1997) obtained 2.5% in subsp. *badia* and 11.22% in *J. cedrus*.

We found the separation method based on mature cone floatability to be satisfactory for *J. oxycedrus* subsp. *macrocarpa*, although it has been used in other taxa such as *J. cedrus* and *J. oxycedrus* subsp. *badia* (Jordan de Urríes, 1997). However, our results on seed selection by the floatability method showed it to be a very efficient method that eliminates the high percentage of floating seeds, most of which lack embryos (Tab. 8), although, as previously indicated, the proportion of viable potential embryos is lower than 12%.

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