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The in vitro morphogenetic capacity of olive embryo explants at different developmental stages, as affected by L-Glutamine, L-Arginine and 2,4-D

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The role of exogenously applied L-glutamine (Gln) and L-arginine (Arg) in growth (dry weight), cell division (callus induction) and differentiation (spherical structure formation), of cotyledonary explants of olive embryos, cv. Chondrolia Chalkidikis, at three developmental stages, and of fully developed embryos, was investigated, in relation to the presence of auxin in the nutrient medium. The percentage of explants forming spherical structures increased with the advancement of the embryo development and was particularly high in the absence of auxin. Histological examination showed these structures to be either somatic embryos at an early stage of development or organ initials. The presence of Arg generally inhibited growth and morphogenetic capacity of explants. Glutamine up to 500 mg.L^{-1} did not affect growth but in most cases improved slightly callus induction. There was a significant, negative correlation between the cumulative concentration of the two amino acids and the parameters measured.

Key words: amino acids, *Olea europaea* L., embryo, in vitro.

Abbreviations: Arg, arginine; Gln, glutamine (Glutamic acid γ-amide); BA, benzyladenine; 2,4-D, 2,4 dichlorophenoxyacetic acid; dAFB, days after full bloom.

INTRODUCTION

Despite genetic diversification of olive species and its great economic importance, certain aspects of developmental physiology, organogenesis and embryogenesis among species, have not been adequately investigated (Rugini, 1986; Rugini, 1988; Mencuccini & Rugini, 1993; Shibli *et al.*, 2001). Generally, in vitro morphogenesis in olive seems to be difficult, since it lies below 50% and is cultivar dependent (Rugini, 1988; Leva *et al.*, 1995, Shibli *et al.*, 2001). Somatic embryogenesis has been achieved from immature zygotic olive embryos, in a medium containing auxin and cytokinin in low concentrations (Rugini, 1988). Immature somatic embryos of cv. Chondrolia Chalkidikis showed low or no morphogenetic capacity when cytokinin was absent, while the presence of auxin (2,4-D 0.5 mg.L^{-1}) inhibited any morphogenetic activity (unpublished data). In other olive cultivars, a positive effect of cytokinin on embryogenetic behavior has been also observed, while auxin had a rather inconclusive effect (Leva *et al.*, 1995; Shibli *et al.*, 2001). Exogenously applied auxin induced changes in endogenous polyamine level, but it was not sufficient to influence root and shoot formation of leafy spurge (Davis, 1997).

Factors promoting in vitro regeneration in olive, such as organic additives, growth regulators and amino acids, should be identified and studied, as has been done for other species (Behrend & Mateles, 1975). Among amino acids involved in plant cell growth and differentiation, L-arginine and L-glutamine seem to play a distinct role, the former as a precursor of polyamine biosynthesis (Minocha & Minocha, 1995), and the latter as a key molecule in nitrogen metabolism (Khlifi & Tremblay, 1995). Considering the ability of olive seeds to produce ethylene (Rinaldi, 2000) and the antagonistic relation

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between ethylene and polyamine biosynthesis, it is interesting to investigate the effect of arginine on the organogenetic ability of cotyledonary explants of olive.

Nitrogen is a crucial factor in growth and morphogenesis of the in vitro cultured plant cells (Reinert *et al.*, 1967; Khlifi & Tremblay, 1995). In callus cells of pine, it is present in an organic form, especially as glutamine and asparagine (Durzan & Chalupa, 1976). According to Reinert *et al.* (1967) it is the concentration rather than the form of nitrogen in the growth medium which is important for embryogenesis of carrot cambial cells. By supplementing nutrient media with mixtures of amino acids or hydrolyzed proteins, growth is frequently enhanced (Behrend & Mateles, 1975), although single amino acids may have the opposite effect (Gamborg, 1970; Behrend & Mateles, 1975). Specifically, alanine, arginine and glutamine either did not affect or inhibited slightly the growth of tobacco, tomato, carrot and soybean cell cultures. On the other hand, soybean cells did not grow on nitrate, unless ammonium or glutamine was also added in the medium (Gamborg, 1970). Furthermore, in mature somatic embryo tissues of black spruce, L-glutamine increased embryogenesis two-fold (Khlifi & Tremblay, 1995) and maintained cell division in Douglas-fir suspension culture (Kirby, 1982).

In this work the morphogenetic efficiency of cotyledonary explants of olive embryos at various developmental stages was studied, in relation to exogenously applied amino acids (L-glutamine and L-arginine) and auxin (2,4-D). The results may contribute to our understanding of certain physiological aspects of morphogenesis related to nitrogen metabolism and to our knowledge of olive regeneration.

MATERIALS AND METHODS

Plant source and culture conditions

Olive embryos, cv. Chondrolia Chalkidikis, at different developmental stages, were used. Seeds of developing fruit, immediately after collection, were surface-sterilized by dipping into 3% sodium hypochlorite solution for 15 min and then thoroughly washed with sterile distilled water, followed by aseptical excision of embryos. The embryo axis and the distal part were removed and the cotyledons were cut transversely to give two explants each. In another experiment, seeds of mature fruits, dried and stored for 4 months at room temperature, were hydrated after sterilization by placing them on a double layer of sterile paper in a 100 mm petri dish with 5 ml of sterile distilled water, overnight, at 23°C, in the dark.

The explants were cultured on half strength MS nutrient medium (Murashige & Skoog, 1962), solidified with 0.7% agar, supplemented with 3% sucrose, with the appropriate growth regulators and amino acids and sterilized at 121°C for 20 min. The pH of the medium was adjusted to 5.7 before autoclaving. The cultures were placed in 16h photoperiod (cool white fluorescent lamps at 30 μmol m^{-2} s^{-1}) and 23°C. Treatments, including at least 10 petri dishes with 4 explants each, were arranged according to a randomized complete-block design.

Experiments with developing embryos

Explants from embryos at three developmental stages: 60, 90 and 125 days after full bloom (AFB), were cultured on media containing 1 $mg.L^{-1}$ (4.44 μM) benzyladenine (BA), 0.5 $mg.L^{-1}$ (2.26 μM) 2,4-D and combinations of L-glutamine (glutamic acid γ-amide, Gln) (0, 500 or 1000 $mg.L^{-1}$) and L-arginine (Arg) (0, 500 or 1000 $mg.L^{-1}$).

Experiments with developed embryos

Explants from embryos of mature fruits collected 150 days AFB and stored for four months at room conditions, were cultured on media containing 1 $mg.L^{-1}$ (4.44 μM) BA, 0.5 $mg.L^{-1}$ (2.26 μM) 2,4-D and combinations of Gln (0, 500, 1000 or 1500 $mg.L^{-1}$) and Arg (0, 500 or 1000 $mg.L^{-1}$). The same experiment was repeated without the addition of 2,4-D.

Measurements – Observations – Histological study

After 30 days of culture, the dry weight of the explants was recorded and the percentages, on a surviving explant basis, of those forming callus or spherical structures (possibly pro-embryos or related structures), were determined.

Each sampling day and at the harvest day (150 days AFB) the embryo length in a 50-fruit sample was measured.

For the histological study, explants were fixed in a formalin/propionic acid/alchohol solution and dehydrated in an ethanol/n-butanol series. Specimens, embedded in paraffin wax, were sectioned at 20 μm with a rotary microtome, stained according to the Safranin-Fast green FCF procedure (Berlyn & Miksche, 1976) and examined for the kind of tissue they were consisted of.

RESULTS

Histological examination of spherical structures

Spherical structures, when formed, appeared in masses along the vascular system (Fig. 1) and on the cut surfaces of the explants but not on callus, i.e. only direct morphogenesis was achieved. They closely resembled spheroblasts of mature trees. These structures increased in size but did not develop into distinct organs at the first stage of culture.

The histological examination revealed an internal structure varying from somatic embryos of globular (Fig. 2a) or torpedo stage (Fig. 2b) to globular masses of callus with a core of organized cells closely resembling organ initials (Fig. 2c).

Experiments with developing embryos

The size measurements showed that the embryos had almost completed their morphological development 90 days AFB (Fig. 3). With the advancement of embryo development, the percentages of both callus and spherical structures were increased. Explants from embryos at the third developmental stage (125 days AFB) exhibited a higher capacity for dry matter accumulation, callus induction and spherical structure formation (Table 1).

Arginine exerted a negative effect on explant growth (dry weight), cell division (callus induction) and differentiation (spherical structure formation). The effect of Gln was inconclusive. However, at its lower concentration (500 mg.L^{-1}), in the absence of

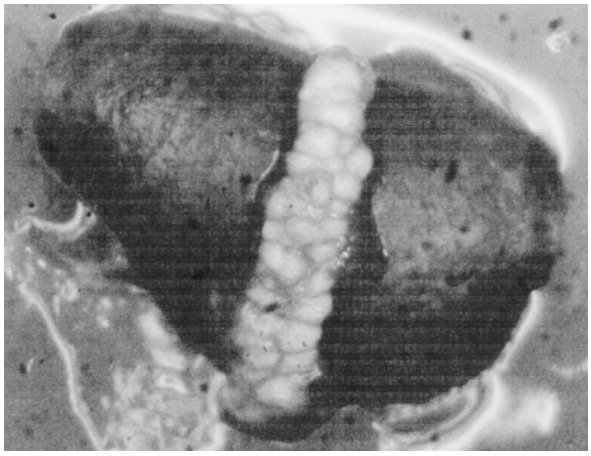

FIG. 1. Masses of spherical structures, formed along the vascular system of a cotyledonary explant from olive embryo (x10).

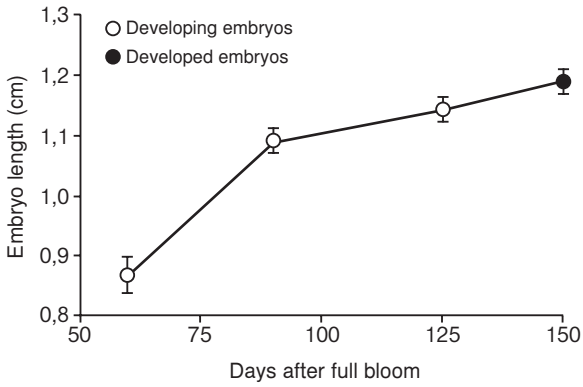

FIG. 3. Time-course of olive embryo growth. Bars represent SE of the mean (n=50).

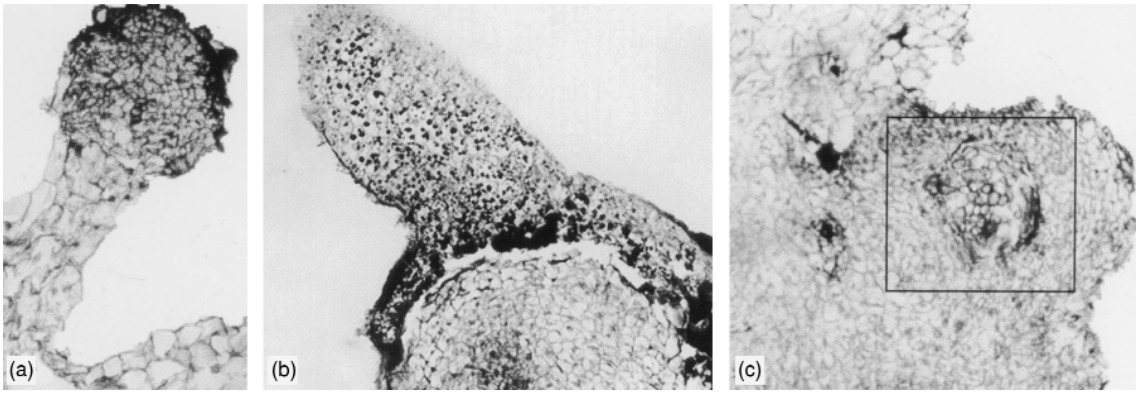

FIG. 2. Somatic olive embryo at the (a) globular or (b) torpedo stages of development; (c) organ initials, consisted of a callus mass with an organized group of cells (x100).

TABLE 1. Effects of glutamine and arginine on dry weight, callus induction and spherical structure formation of explants from olive embryos at three developmental stages

	Glutamine (mg.L^{-1})	Arginine (mg.L^{-1}) 0	500	1000
Dry weight (mg)				
60 d AFB[1]	0	5.08	3.40	2.28
	500	6.63	3.50	1.36
	1000	3.25	2.70	2.30
90 d AFB	0	4.30	3.42	3.28
	500	4.53	3.7	3.45
	1000	4.36	3.32	2.96
125 d AFB	0	6.90	5.06	5.40
	500	7.88	6.83	5.55
	1000	6.83	5.79	3.75
Percentage of explants with callus induction				
60 d AFB	0	37.4	25.0	3.6
	500	87.4	25.0	6.2
	1000	31.2	28.1	6.2
90 d AFB	0	40.6	25.0	14.3
	500	40.6	25.0	9.5
	1000	40.0	17.9	9.1
125 d AFB	0	57.5	47.5	29.6
	500	72.5	30.0	25.0
	1000	77.3	30.0	10.0
Percentage of explants with spherical structures				
60 d AFB	0	6.2	0.0	0.0
	500	3.1	0.0	0.0
	1000	0	0.0	0.0
90 d AFB	0	6.2	0.0	0.0
	500	3.1	0.0	0.0
	1000	5.0	0.0	0.0
125 d AFB	0	12.5	0.0	6.75
	500	7.5	5.0	0.0
	1000	9.1	5.0	0.0

dAFB: days after full bloom
For statistical analysis see Table 2

Arg, Gln either did not affect or slightly improved dry weight and callus induction but decreased spherical structure formation in explants from embryos at all developmental stages (Table 1). A significant negative correlation between Arg and the three parameters measured at all three developmental stages was evident. The cumulative concentration (in mM) of the two amino acids was also negatively correlated with dry weight and callus induction (Table 2).

Experiments with developed embryos

In the presence of auxin, Arg suppressed dry matter accumulation regardless of Gln concentration (Table 3). Glutamine, when alone, had a negative effect on dry weight, proportional to its concentration when auxin was present (Table 4). All combinations of the two amino acids resulted in a decrease at the dry weight of explants. Such effects were not observed on a medium without auxin. The highest dry weight values occurred on a medium with auxin, supplemented or not with 500 mg.L^{-1} Gln and without Arg (Table 3). A significant negative correlation between the dry weight and the cumulative concentration of the two amino acids in the medium, was also evident (Table 4).

Callus induction occurred only in the presence of auxin. Glutamine at its lowest concentration (500 mg.L^{-1}), combined with auxin, increased callus induction only slightly. Arginine alone or in combination with Gln inhibited callus formation. Appreciable percentages of explants forming spherical structures were determined only on a medium free of auxin and Arg, with the highest value observed when both amino acids were absent (Table 3).

TABLE 2. Linear correlation coefficient (r) between Gln, Arg or their cumulative concentration (mM) in the nutrient medium and the dry weight of explants, the percentage of explants with callus induction and the percentage of explants with spherical structures. Explants originated from embryos at three developmental stages (60, 90 and 125 days after full bloom)

	Days after full bloom 60		90		125	
Dry weight (mg)						
Gln	-0,150	ns	-0,120	ns	-0,110	ns
Arg	-0,640	***	-0,500	****	-0,500	****
Gln+Arg	-0,510	***	0,365	**	-0,370	**
n	50		53		71	
Percentage of explants with callus induction						
Gln	+0,003	ns	-0,142	ns	-0,156	ns
Arg	-0,793	*	-0,974	****	-0,900	***
Gln+Arg	-0,455	ns	-0,672	*	-0,642	ns
n	9		9		9	
Percentage of explants with spherical structures						
Gln	-0,408	ns	-0,069	ns	-0,168	ns
Arg	-0,612	ns	-0,823	***	-0,728	*
Gln+Arg	-0,680	*	-0,526	ns	-0,552	ns
n	9		9		9	

Gln, Arg: L-glutamine and L-arginine, respectively
*, **, ***, ****: P<5 $^{o}/_{o}$, 1 $^{o}/_{o}$, 1 $^{o}/_{oo}$, 1 $^{o}/_{ooo}$, respectively; ns: non significant
n: number of counts (explants for d.wt., or petri dishes for the percentages)

TABLE 3. Effects of glutamine and arginine on dry weight, callus induction and spherical structure formation of explants from fully developed olive embryos, cultured on media with (0.5 mg.L-1) or without auxin (2,4-D). No callus was induced on the medium without auxin

	Glutamine ($mg.L^{-1}$)	Arginine ($mg.L^{-1}$) 0	500	1000
	Dry weight (mg)			
+ auxin	0	16.74	11.08	6.00
	500	15.20	6.71	5.64
	1000	9.30	4.30	5.98
	1500	6.63	4.43	5.90
No auxin	0	11.90	7.38	5.98
	500	7.80	10.40	8.60
	1000	8.46	9.35	5.70
	1500	10.51	9.89	7.55
	Percentage of explants with callus induction			
+ auxin	0	81.0	27.8	8.3
	500	100.0	26.2	13.3
	1000	25.0	0.0	0.0
	1500	0.0	0.0	0.0
	Percentage of explants with spherical structures			
+ auxin	0	10.7	5.6	0.0
	500	0.0	0.0	0.0
	1000	20.0	0.0	0.0
	1500	0.0	0.0	0.0
No auxin	0	53.8	0.0	6.7
	500	33.3	8.3	0.0
	1000	37.5	0.0	0.0
	1500	40.0	0.0	0.0

For statistical analysis see Table 4

TABLE 4. Linear correlation coefficient (r) between Gln, Arg, or their cumulative concentration (mM), in media with (0.5mg.L-1) or without auxin (2,4-D) and the dry weight of explants, the percentage of explants with callus induction and the percentage of explants with spherical structures. Explants originated from fully developed embryos (150 days after full bloom)

	Nutrient Medium + auxin		no auxin	
	Dry weight (mg)			
Gln	-0,500	****	0,055	ns
Arg	-0,560	****	-0,380	**
Gln + Arg	-0,665	****	-0,120	ns
n	52		50	
	Percentage of explants with callus induction			
Gln	-0,543	ns	-	
Arg	-0,588	*	-	
Gln + Arg	-0,750	**	-	
n	12		-	
	Percentage of explants with spherical structures			
Gln	-0,178	ns	-0,127	ns
Arg	-0,520	ns	-0,838	***
Gln + Arg	-0,394	ns	-0,492	ns
n	12		12	

Gln, Arg: L-glutamine and L-arginine, respectively
*, **, ***, ****: P<5 ‰, 1 ‰, 1‰, 1 ‰, respectively; ns: non significant
n:number of counts (explants for d.wt., or petri dishes for the percentages)

Summarizing the effects of auxin, it should be pointed out that its role in dry matter accumulation appeared rather inconclusive. On the other hand, its presence in the medium was a prerequisite for callus induction, which could give rise to indirect morphogenesis, though it depressed spherical structure formation.

DISCUSSION

Embryos of cv. Chondrolia Chalkidikis persistently complete their morphological development and reach their final size about 90 days AFB (Voyiatzis & Pritsa, 1994). However, they continue their physiological development past this limit. Our results showed that the capacity of embryos to form callus and spherical structures increased with physiological age. Embryos did not lose their morphogenetic ability even after storage and dehydration of the seeds. The change in the physiological status of the embryos was manifested by an increase in their amino acid tolerance. This may have been brought about by a decrease in the free amino acid level, resulting in a depletion of the endogenous amino acid pool, as it happens in developing pea cotyledons (Lanfermeijer *et al.*, 1989).

The highest morphogenetic ability of embryo explants, for all olive cultivars tested so far, has been observed between 60 and 90 days AFB (Rugini, 1988; Leva *et al.*, 1995). Our results showed that in 'Chondrolia Chalkidikis' this ability continues to increase beyond this point and reaches its highest value after the embryos have completed their morphological and physiological development. Explants of fully developed embryos proved to be more efficient in direct morphogenesis, in the absence of auxin. Direct organogenesis in cotyledonary explants of other plant species was favored under similar conditions, with BA as a sole growth regulator in the medi-

um (Lee *et al.*, 2003, Yancheva *et al.*, 2003). On the other hand, the callus formed, in the presence of auxin, could be the initial stage of indirect morphogenesis, as previously reported for cotyledonary and other explants of 'Nabali' olive (Shibli *et al.*, 2001).

The formation of spherical bodies in masses was not favored by the addition of amino acids in the medium, in general. These structures resembled in appearance spheroblasts (knobby-like protuberances known as ovuli and consisted of meristematic tissue), which are formed on the branches of mature olive trees. Their histological examination showed that they consist of organized cell groups closely resembling organ initials or embryoids at the globular or torpedo stage of development. Globules with varied internal structure on callus and embryonic masses on cotyledonary explants have also been observed in other olive cultivars (Rugini, 1988; Leva *et al.*, 1995).

Arginine reduced growth and minimized callus initiation and spherical structure formation. A similar negative effect of Arg on cell growth has not been detected in other plant species (Behrend & Mateles, 1975). On the contrary, Arg at 500 $mg.L^{-1}$ slightly improved growth of axillary buds of olive single-node explants (Rugini, 1984).

Glutamine, at its lowest concentration, did not affect or slightly enhanced growth and cell division of explants from embryos at all developmental stages. Generally, Gln is relatively nontoxic and improves growth in many species, while other amino acids do not (Reinert *et al.*, 1967; Gamborg, 1970; Behrend & Mateles, 1975; Rugini, 1984; Khlifi & Tremblay, 1995). This beneficial effect has been explained on the basis that Gln offers a readily available source of nitrogen, providing the necessary carbon skeletons and ammonium, both being limiting factors for cell growth (Gamborg, 1970). Accordingly, the low embryogenetic capacity of zygotic embryos cultured on modified 'OM medium' was attributed to the low content of nitrogen in its reduced form (Rugini, 1984; Rugini, 1988).

The presence of both Gln and Arg in the medium generally had an inhibitory effect on all parameters measured. In all experiments, a negative and highly significant correlation between the cumulative concentration of the two amino acids and the dry weight of the explants was observed. This differs from reports that in similar cases amino acids exerted an inhibitory effect when acted one at a time while their mixtures did not (Gamborg, 1970; Behrend & Mateles, 1975). However, it is in agreement with the concept that a high concentration of amino acids might be inhibitory due to the accumulation of ammonium ions, in addition to their osmotic role (Khlifi & Tremblay, 1995).

In our experiments, addition of auxin in the medium inhibited direct organogenesis, but enhanced callus formation (indirect morphogenesis) in olive embryo explants. Whether this inhibition might be attributed to the disruption exogenous auxins cause to auxin gradients necessary for polarized growth (Nissen & Minocha, 1993), or to the interference of auxins with polyamine or other growth regulator biosynthesis (Davis, 1997), remains to be clarified.

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