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Title: Influence of carbon source and concentration on the *in vitro* development of olive (*Olea europaea* L.) zygotic embryos and explants raised from them.

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ABSTRACT

The influence of sucrose or mannitol on *in vitro* zygotic embryo germination, seedling development and explant propagation of olive tree (*Olea europaea* L.) was compared. Embryos germinated without sucrose in the medium but for adequate development of the seedlings to yield viable plants, a carbohydrate supply was necessary; both sucrose and mannitol were equally suitable for this purpose. However, when explants obtained from *in vitro* germinated embryos were cultured with mannitol or sucrose, then the polyalcohol promoted significantly higher growth than sucrose by increasing shoot length, pairs of leaves formed, and breaking apical dominance. This improved the *in vitro* culture of olive plant material, thus allowing new olive clonal lines to be obtained in shorter times. This will assist in future breeding experiments with the species.

Key words: *Olea europaea* L., bud explants, embryo germination, *in vitro* culture, mannitol, sucrose.

INTRODUCTION

The olive tree (*Olea europaea* L.) has a long juvenile phase so that in some cases it can take more than 10 years to reach flowering (Natividade, 1957; Rugini, 1986; Bellini, 1992). Moreover, germination of the olive seeds is, under field conditions, a slow and rare event, and is impractical for the propagation of new breeding plants. Therefore, to establish olive breeding programs, it is first necessary to speed up the germination of the seeds and the further development of the seedlings to shorten the juvenile stage.

By means of *in vitro* olive zygotic embryo germination with an appropriate nitrogen supply, a reduction of the germination time, a germination percentage close to 100% and an increase of the growth rate of seedlings has been achieved (García et al., 1994; Sarmiento et al., 1994; Acebedo et al., 1997). Nevertheless, olive germinated *in vitro* still shows a limited proliferation rate due mainly to a lack of secondary shoot production; this technique needs further improvement.

In vitro cultured plants and tissues need an exogenous supply of carbohydrates as source of carbon because such plants are not fully autotrophic. In general, sucrose is the carbohydrate of choice as carbon source for *in vitro* plant culture, probably because it is the major transport-sugar of many plants (Murashige and Skoog, 1962; Thorpe, 1982; Thompson and Thorpe, 1987). However, there are a number of species that can grow on carbohydrates different than sucrose (Pua and Chong, 1984; Nadel et al., 1989; Marchal et al., 1992; Vu et al., 1993). In many cases, those alternative carbohydrates are sugar alcohols, such as sorbitol, glycerol or mannitol.

Sugar alcohols are primary photosynthetic products that fulfill the functions of reduced carbon translocation and storage in a wide range of plant species (Steinitz, 1999). Sorbitol, a polyol that occurs abundantly in species of the Rosaceae, has been

shown to be a good carbon substrate for cultures of *Malus* species and *Prunus persica* (Chong and Taper, 1972) and was able to support growth of 11 Rosaceae species (Coffin et al., 1976). Mannitol, the most widely distributed sugar alcohol in the plant kingdom (Bieleski, 1982), can substitute for sucrose in callus cultures of *Fraxinus americana* (Wolter and Skoog, 1966) and cell suspension cultures of celery (Stoop and Pharr, 1993), both species that produce and translocate mannitol. Therefore, it is probable that the ability of cultured cells and tissues to grow on mannitol is related to the capacity of the species to form and translocate this polyol. Mannitol utilization in vascular plants occurs at low rates in suspension cultures of Monterey pine and carrot (Thompson et al., 1986) and at higher rates in leaves of white ash, lilac and celery (Trip et al., 1964; Fellman and Loescher, 1987).

In olive, mannitol is a major product of photosynthesis that reaches high concentrations in leaves that are second only to those of glucose and is translocated in the phloem (Flora and Madore, 1993). For this reason, this polyalcohol could be a suitable carbon source for the *in vitro* culture of olive tissues. Leva et al. (1994; 1995) have reported that mannitol improved the *in vitro* propagation of agamic olive explants, collected from mature trees growing in the field.

The aim of this work was to compare the influence of sucrose and mannitol on *in vitro* germination and development of isolated zygotic embryos of olive as well as on the growth of the shoots obtained in order to improve the *in vitro* propagation potential of olive.

MATERIALS AND METHODS

Fruits from open pollinated *Olea europaea* L. var Manzanillo were harvested at the yellow–green-violet color transition. The stones were separated from the mesocarp (flesh) and the sclerified endocarp was broken (Sotomayor-León & Caballero, 1990).

The stoneless seeds were then surface sterilized using both ethanol and sodium hypochlorite and soaked in sterile water for 48 h at 25 °C as described by Sarmiento et al. (1994). Embryos were then isolated by cutting off the two lateral sections of the endosperm and freeing the embryo from the remaining seed tissues.

For germination, the isolated embryos were placed individually in sterile test tubes (21x150 mm) with 8 ml of culture medium of half-strength Olive Medium (OM) (Rugini, 1984) without growth regulators and different concentrations of sucrose or mannitol. Forty-eight embryos were cultured for each sugar concentration tested.

The tubes were closed with plastic caps and sealed with parafilm and placed in a growth chamber at 23 ± 2 °C with a light intensity of 30 $\mu E.m^{-2}.s^{-1}$ provided by F38W/133ST Cool White fluorescent tubes with a 16-h photoperiod. After 60 d culture, percent of germination, shoot and root length of the seedlings, number of nodes and percent of viable plants were recorded. Olive seedlings were transferred and acclimated to *ex vitro* conditions as described by García et al. (1999).

From the seedlings raised via embryo germination, uninodal single lateral-bud explants were prepared by removing one of the two axillary buds of the uninodal explants. These explants were then propagated *in vitro* on full strength OM medium, plus 1 $mg.l^{-1}$ zeatin and 30 $g.l^{-1}$ sucrose. After a few subcultures, a clonal population of non-rooted shoots was harvested.

Three experiments were conducted with this material. In the first one, uninodal single-bud explants were cultured for 60 d on full strength OM medium, plus 1 $mg.l^{-1}$ zeatin and supplied with 3 different concentrations of sucrose or mannitol (7.5, 15 or 30 $g.l^{-1}$). Twenty-four explants were used for each sugar concentration. This experiment was repeated 4 times.

The second experiment was similar to the first one except that uninodal explants with two axillary buds instead of one axillary bud were used and that the experiment was repeated twice.

For the third experiment, 24 single-bud explants obtained in media with 15 $g.l^{-1}$ of sucrose or mannitol were subcultured on media containing the same or the other carbohydrate at 30 $g.l^{-1}$.

In all the cases, the cultures were placed in a growth chamber under the same conditions as described for embryo germination. After a 60 d culture period, shoot length, number of nodes formed and number of shoots per explant were recorded.

For chlorophyll estimations, 1g of seedling leaves were frozen in liquid nitrogen, ground to a powder, extracted with 80% (v/v) acetone in the dark and centrifuged at 15000 x g for 10 min. Absorbance at 664 and 647 nm was recorded and the extinction coefficients of Graan and Ort (1984) were used for determination of chlorophyll a, b and total chlorophyll.

Statistical significance was determined using analysis of variance according to the least significant difference method at $P \leq 0.05$ level.

RESULTS

In the first experiment, the influence of the sucrose concentration on the germination and development of the olive embryos was tested (Table 1). The first steps of germination (opening and greening of the cotyledons and first appearance of the radicle and shoot) were not influenced by the presence of sugar in the medium. In all cases, a 100% of germination rate was reached, but further development of the seedlings depended on the presence of sucrose. When no sugar was supplied to the embryos, the growth after 60 days of culture was poor yielding no viable plants suitable for *ex vitro*

transfer. However, the addition of sucrose to the medium allowed 85-87% of the seedlings to develop viable plants at 15-20 $g.l^{-1}$ concentrations.

When mannitol was used for the germination of the embryos, the results were similar to those with sucrose, and the embryos germinated and grew equally well with both carbohydrates. Thus, germination and early development of the isolated embryos was independent of the type of carbon. Also, no difference was found when the seedlings were transferred to outside conditions. Seedlings cultured in sucrose or mannitol could be acclimated to *ex vitro* conditions with about 90% success.

The main difference between sucrose and mannitol influence on the germination and development of the olive embryos was found in the chlorophyll contents of the seedlings leaves, which were consistently higher in those cultured on mannitol (Table 2).

The *in vitro* propagation of olive shoots was strongly influenced by the carbon source employed. Mannitol was much more efficient than sucrose in promoting growth of single-bud explants (Table 3). Although increasing sucrose concentration from 7.5 to 15 or 30 $g.l^{-1}$ resulted in greater shoot length, number of nodes and internode length, no statistically significant differences were found among the 3 sucrose concentrations tested. Use of mannitol at 7.5 $g.l^{-1}$ resulted in growth similar to that produced by sucrose at higher concentrations but when the mannitol supply was raised to 15 or 30 $g.l^{-1}$, the shoot length and number of nodes were significantly higher than was obtained with similar concentrations of sucrose. With the polyalcohol, a significantly greater shoot length and internode length was obtained with 30 $g.l^{-1}$ than with 15 $g.l^{-1}$.

When explants with two-axillary buds were used, the mannitol stimulated the sprouting of both buds, giving raise to two shoots per explant at all concentrations tested. With sucrose, this occurred only at 7.5 $g.l^{-1}$ and higher concentrations of this

sugar resulted in significantly fewer shoots per explant (Table 4). The length of each individual shoot produced by the two-axillary buds explants was diminished compared to the results obtained with single-bud explants (Table 3) probably due to competition between the two shoots. However, if the total shoot growth (sum of the lengths of the shoots produced by each explant) and the total number of new nodes formed per explant are considered, the growth of the two-bud explants was higher, except when 30 $g.l^{-1}$ of sucrose was used. Again, mannitol produced more growth than sucrose, even at 7.5 $g.l^{-1}$.

Table 5 shows the results of an experiment where single-bud explants obtained in sucrose or mannitol were subcultured in media containing the same or the other carbohydrate at 30 $g.l^{-1}$. The explants obtained in mannitol and recultured again with the same sugar presented significantly better growth than the other treatments, particularly when compared to those supplied always with sucrose. When the explants grown in sucrose were transferred to mannitol, the growth was significantly stimulated in relation to those recultured again on sucrose. On the other hand, the explants obtained in mannitol and transferred to sucrose diminished their growth rate but grew significantly more than the explants supplied only with sucrose.

DISCUSSION

In vitro olive embryo germination has become a powerful tool to overcome the problems derived from the low ability of the seeds to germinate in the field (García et al., 1994; Sarmiento et al., 1994; Acebedo et al., 1997), and thus to speed up the process of obtaining new olive plants for olive breeding programs.

Our results show that the *in vitro* germination of isolated olive embryos is independent of the presence of a carbon source, since the diverging and greening of the cotyledons, as well as the first appearance of shoot and root primordia takes place even in the absence of sugar in the media (Table 1). Thus, the 100% of germination achieved

under these conditions indicates that the energy needed for germination *in vitro* is provided by the reserves stored in the embryo. Cañas et al. (1987) had observed this previously for whole olive seeds cultured *in vitro* but it is worth to note that in our case the endosperm is not present.

Nevertheless, further development of the germinated seedling required the presence of sucrose and when no sugar was supplied (Table 1), the growth of the embryos stopped after a few days.

The use of mannitol as carbon source proved to be as efficient as sucrose for the *in vitro* germination and development of the olive embryos. This meant that the olive seedlings could metabolize mannitol to grow. The main differences were found in relation to the chlorophyll contents, which were clearly higher in the seedlings supplied with mannitol (Table 2). This may indicate that a healthier photosynthetic system is induced when the polyalcohol is the source of carbon. A similar stimulation of chlorophyll biosynthesis has been described for citrus somatic embryos growing on glycerol as sole carbon source (Vu et al., 1993).

These results show that a source of carbon is required for the germinated embryos to grow properly, although at this initial stage no specific preference for sucrose or mannitol was found. A much different behavior was observed when the seedlings obtained from the *in vitro* germinated embryos were propagated. In that case, the explants showed a clear preference for the mannitol (Table 3). The addition of 15 or 30 $g.l^{-1}$ of mannitol to the medium produced double the shoot length than was obtained at similar concentrations of sucrose and the number of nodes was also significantly higher. Similar results have been reported by Leva et al. (1994; 1995) for *in vitro* established uninodal explants taken from adult olive plants of cvs. Frangivento and Maurino, growing in the field.

This effect of the mannitol was also evident when two-bud explants were propagated and only mannitol stimulated both buds to sprout (Table 4). Consequently, the total length of both shoots on the explant and the total number of nodes per explant was higher than in the single-bud explants (Table 3). This is remarkable because olive is characterized by strong apical dominance *in vitro*, with the formation of secondary axillary shoots being scarce, thus limiting the *in vitro* micropropagation potential. Clearly, the multiplication rate of the olive explants *in vitro* is enhanced by the use of mannitol.

Moreover, the positive effect of this carbohydrate on the development of the olive explants *in vitro* was persistent in further subcultures, even when mannitol-grown explants were transferred to media with sucrose (Table 5).

Mannitol has been widely used to apply osmotic stress *in vitro* when added to media at high concentrations. Since it was believed to be nearly metabolically inert, the *in vitro* mannitol-dependent phenomena are usually defined predominantly as osmotic effects (Steinitz, 1999). However, in species that form mannitol photosynthetically and that can grow *in vitro* on it as well as on sucrose at similar concentrations, the osmotic effect would be far less significant.

Mannitol is a major photosynthetic product in the olive tree, that is translocated by the phloem (Flora and Madore, 1993). Leva et al. (1995) found that it is incorporated by the explants *in vitro*, reaching high concentrations in the tissues and that it is metabolized to other carbohydrates. Why mannitol promotes growth of the olive explants *in vitro* more efficiently than sucrose is not known.

In celery, the anabolic and catabolic pathways of mannitol metabolism have been elucidated (Rumpho et al., 1983; Stoop and Pharr, 1992). Pharr et al., (1995) have suggested that the metabolic use of mannitol provides some energetic advantage to the

plants, since the hexose-P generated from mannitol in sink cells is accompanied by the net generation of two ATPs per mannitol converted. In contrast, the initial generation of hexose-P from sucrose in sink cells occurs at the expense of ATP. The same could be the case in olive tree metabolism.

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Table 1. Germination and development of olive embryos according to sucrose concentration in the culture medium, after 60 days of culture. Data are mean of 48 embryos per test. In each row, different letters indicate significant differences at $p \leq 0.05$.

	Sucrose ($g.l^{-1}$)				
	0	10	15	20	25
% Germination	100^a	100^a	100^a	100^a	100^a
Shoot length (mm)	3^a	20^b	28^b	27^b	24^b
Root length (mm)	10^a	70^b	139^c	138^c	110^c
% Viable plants	0^a	70^b	87^b	85^b	73^b

Table 2. Influence of the type of sugar (mannitol or sucrose) on the chlorophyll content of seedling leaves, after 60 days of *in vitro* culture.

	Mannitol ($15\ g.l^{-1}$)	Sucrose ($15\ g.l^{-1}$)
Chl a (µg/g fw)	180.19	37.34
Chl b (µg/g fw)	103.85	26.17
Total chl (µg/g fw)	284.04	63.51

Table 3. Effect of carbohydrate type on the *in vitro* growth of single-bud olive explants, after 60 days of culture. Data are mean of 4 subcultures (24 explants per sugar concentration in each subculture). In each row, different letters indicate significant differences at $p \leq 0.05$.

	Sucrose ($g.l^{-1}$)			Mannitol ($g.l^{-1}$)		
	7.5	15	30	7.5	15	30
Shoot length (mm)	16.5^{a}	23.5^{a}	23.0^{a}	20.8^{a}	44.8^{b}	53.6^{c}
Nodes/shoot	3.5^{a}	4.3^{a}	4.2^{a}	4.5^{a}	6.9^{b}	6.8^{b}
Internode length (mm)	4.6^{a}	5.4^{ab}	5.5^{ab}	4.5^{a}	6.5^{b}	7.9^{c}

Table 4. Effect of carbohydrate type on the *in vitro* growth of two-bud olive explants, after 60 days of culture. Data are mean of 2 subcultures (24 explants per sugar concentration in each subculture). In each row, different letters indicate significant differences at $p \leq 0.05$.

	Sucrose ($g.l^{-1}$)			Mannitol ($g.l^{-1}$)		
	7.5	15	30	7.5	15	30
Shoots/explant	2.0^{c}	1.6^{ab}	1.4^{a}	1.9^{bc}	2.0^{c}	2.0^{c}
Shoot length (mm)	11.1^{a}	16.4^{ab}	8.6^{a}	24.2^{cd}	21.3^{bc}	31.3^{d}
Total shoot growth (mm)	22.1^{ab}	26.6^{ab}	12.0^{a}	45.0^{c}	42.6^{bc}	62.6^{d}
Nodes/shoot	2.9^{a}	3.2^{a}	2.0^{a}	5.2^{b}	5.2^{b}	5.0^{b}
Nodes/explant	5.7^{a}	5.3^{a}	2.8^{a}	9.7^{b}	10.4^{b}	10.0^{b}
Internode length (mm)	3.7^{a}	5.3^{bc}	5.0^{b}	4.5^{ab}	3.9^{a}	6.1^{c}

Table 5. Effect of changes in carbohydrate on the *in vitro* growth of single-bud explants after 60 days of culture. Data are mean of 24 explants per test. In each column, different letters indicate significant differences at $p \leq 0.05$.

1^{st} culture ($15\ g.l^{-1}$)	2^{nd} culture ($30\ g.l^{-1}$)	Shoot length (mm)	Nodes/shoot	Internode length (mm)
Sucrose	Sucrose	13.4^{a}	4.1^{a}	3.1^{a}
	Mannitol	41.2^{c}	7.5^{b}	5.6^{b}
Mannitol	Sucrose	26.1^{b}	5.0^{a}	5.2^{b}
	Mannitol	49.0^{d}	7.7^{b}	6.3^{c}