BIOLOGIA PLANTARUM 48 (4): 589-592, 2004

BRIEF COMMUNICATION

Micropropagation of a local olive cultivar for germplasm preservation

M. ZACCHINI[1] and M. De AGAZIO

Istituto di Biologia Agroambientale e Forestale, Consiglio Nazionale delle Ricerche, Via Salaria km 29.300,I-00016 Monterotondo Scalo, Roma, Italy

Abstract

In vitro shoot culture was applied to an Italian local cultivar Nebbiara of olive (*Olea europaea* L.) to preserve its endangered germplasm. This cultivar showed a notable difficulty for the *in vitro* establishment due to heavy pathogen contamination. Mercury chloride and sodium hypochloride in the sterilisation step and antibiotics in culture media allowed to overcome the problem. Proliferation of shoot apical bud on olive culture medium with 36 g dm^{-3} mannitol and 4.56 μM zeatin appeared very satisfactory. All the explants tested rooted during a subculture (1 month) preceeded by a 5-d long dark pre-treatment.

Additional key words: axillary buds, culture media, *in vitro* establishment, *in vitro* rooting, nodal segments.

Olive is one of the most important fruit trees cultivated in the Mediterranean basin. It tolerates moderate unfavourable environmental conditions such as high summer temperatures and drought, and it has a good adaptation to different edaphic situations (ranging from heavy and clavey soils to light and sandy ones) and a long life (Rugini *et al*. 2000).

For these characteristics olive cultivars have been cultivated for centuries in difficult agronomic areas, characterised for particular microclimates, where they expressed some peculiar phenotypical traits that, with time, resulted in an establishment of an ecotype. The local cultivars represent an interesting plant material not only for economical aspects but also as possible "gene banks" of biodiversity particularly important for stress tolerance studies.

Many studies dealt with the possibility to use *in vitro* culture methods to preserve endangered local cultivars from disappearance (Bajaj 1983, Grout 1990, Bekheet 2000, Bekheet *et al*. 2002, Gagliardi *et al*. 2002, Wang *et al*. 2002). Recently, the establishment and *in vitro* regeneration of olive has been attempted by some unconventional techniques, such as protoplast technology and haploid culture, to obtain genetic improvements through somaclonal variation (Mencuccini 1991, Rugini *et al*. 2000); Cell and tissue culture techniques, *i.e.* somatic embryogenesis (Leva *et al*. 1995, Rugini and Caricato 1995), micropropagation (Rugini 1984) and callus cultures (Martino *et al*. 1999), have been applied to propagate selected clones or cultivars difficult to root with conventional techniques (Rugini *et al*. 1992, Briccoli Bati *et al*. 1999).

The aim of this work was to evaluate the suitability of the *in vitro* culture technique to propagate and preserve the endangered olive cultivar Nebbiara. This cultivar is characterised by a fast and vigorous growth, a good productivity with a satisfactory fruit quality and a good tolerance to frost and it is cultivated for centuries in a narrow area of central Italy.

Branches of the olive (*Olea europaea* L.) cultivar Nebbiara were collected in winter from seven fifty-year old field-grown trees. Current year shoots were defoliated, cut in 3 cm long nodal segments and washed for 30 min in running tap water. Axillary buds, excised

Received 27 January 2003, *accepted* 7 January 2004.

Abbreviations: MS - Murashige and Skoog; OM - olive medium, BAP - 6-benzylaminopurine; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid.

Acknowledgements: This work was funded by Amministrazione Provinciale di Rieti. Authors wish to thank Prof. E. Rugini for his helpful suggestions, Dr. G. Frappetta for the collaboration in recognising and collecting the plant material and Mr. R. Buffone for technical assistance.

[1] Corresponding author; fax: (+39) 06 9064492, e-mail: massimo.zacchini@ibaf.cnr.it

from nodal segments, and 3 cm long nodal segments were exposed to four different sterilisation procedures: *1* - immersion in 70 % ethanol for 1 min and washing three times in sterile distilled water; *2* - immersion in 70 % ethanol for 1 min, washing in sterile distilled water, then immersion in 15 % sodium hypochloride (7 % of active Cl) for 15 min and washing again three times in sterile distilled water; *3* - immersion in 0.1 % $HgCl_2$ for 5 min, washing in sterile distilled water, then immersion in 15 % sodium hypochloride for 15 min and washing again three times in sterile distilled water; *4* - as in 3 but with antibiotics (0.5 g dm^{-3} carbenicillin and 0.1 g dm^{-3} cefotaxime) added to initial culture medium.

After sterilisation, nodal segments and axillary buds were transferred to initial culture medium consisting in half-strength Murashige and Skoog (1962; MS) medium supplemented with 36 g dm^{-3} mannitol and 4.56 μM zeatin and cultured in glass tubes for a month.

Shoot tips 3 cm-long were collected from established cultures and placed in two proliferation medium for 1 month (subculture) with or without the excision of the apical bud: *A* - olive proliferation medium (Rugini 1984; OM) with 36 g dm^{-3} mannitol, 13.68 μM zeatin, 4.33 μM gibberellic acid (GA_3) and 0.49 μM indole-3-butyric acid (IBA) and *B* - MS proliferation medium with 30 g dm^{-3} sucrose, 8.88 μM 6-benzylaminopurine (BAP), 4.33 μM GA_3 and 0.49 μM IBA.

To evaluate *in vitro* rooting capability of olive culture, shoot tips were placed in OM medium with half-dose macronutrients, 20 g dm^{-3} sucrose and 3.22 μM α-naphthaleneacetic acid (NAA) for 1 month, and subjected or not to 5-d dark pre-treatment.

Culture media were adjusted to pH 5.8 before the autoclaving at 121 °C for 25 min and solidified with 0.8 % agar. Antibiotics were added to culture medium after autoclaving. Cultures were maintained in a growth chamber at 25 °C with a 16-h photoperiod (photon flux density of 40 μmol m^{-2} s^{-1}).

All analyses were performed on at least five replicates. Each replicate consisted in a plastic vessel containing 9 shoots, except for sterilisation procedure. Data obtained were subjected to multiple range analysis of variance (*ANOVA*). Tukey's test was used to compare means.

All chemicals were purchased from *Sigma* (Milan, Italy) except for antibiotics (*Duchefa*, Haarlem, The Netherlands).

The commonly adopted sterilisation procedures, *i.e.* 70 % ethanol or 70 % ethanol followed by 15 % sodium hypochloride, were unsatisfactory in both types of explants. The use of 0.1 % mercury chloride followed by 15 % sodium hypochloride allowed a good percentage of survived explants (25 %), especially when antibiotics were supplied to culture medium (86 %). As expected, nodal segments resulted to be the preferable starting material but also excised axillary buds showed an *in vitro* establishment, generally difficult to obtain in olive (Rugini *et al*. 2000).

To overcome the strong apical dominance (Rugini and Panelli 1993), a stock of the explants were deprived of the apical bud (decapitated shoots). This technique is known to force the sprouting of axillary buds (Rugini *et al*. 2000), supporting cytokinin action, as other *in vitro* culture factors do (Morini *et al*. 1991, Zacchini and Morini 1995). Explants grown on OM medium showed a better proliferation rate than those on MS medium (Table 1). The excision of apical bud promoted a remarkable enhancement of the number of the explants that presented axillary bud outgrowth in both media, confirming that this technique can ameliorate the proliferative capability of *in vitro* olive shoots.

Media composition and apical bud excision notably affected also the length of the shoots grown from axillary buds (Table 1). Shoots grown in OM medium were longer than those grown in MS medium. Plantlets grown on MS medium showed a reduced number of internodes (data not shown). Apical bud excision allowed to obtain 31 % of shoots longer than 3 cm, only in OM medium, indicating these conditions as very suitable for micropropagation of this olive cultivar. Moreover, explants grown in OM medium produced less basal callus, that is another desirable trait for *in vitro* shooting and rooting.

Explants were exposed or not to a 5-d long dark treatment that was reported as beneficial to improve olive *in vitro* rooting (Rugini and Fedeli 1990). Data concerning the percentage of rooting shoots showed a remarkable rooting capability of Nebbiara explants (Table 2). A short period of dark exposure promoted root formation in all the explants tested. On the contrary, the

Table 1. Shoot proliferation and shoot length after a subculture in two different proliferation media (OM, MS) with (-bud) or without apical bud excision (+ bud). Means ± SE. Within raw, letters indicate statistically different values (Tukey's test, $P \leq 0.05$).

	OM		MS	
	-bud	+bud	-bud	+bud
Proliferation [%]	82a	41b	44b	25c
Length [cm]	2.57 ± 0.22a	1.92 ± 0.13b	1.15 ± 0.09c	1.08 ± 0.09c

Table 2. Rooting characteristics of olive shoots, after a subculture, in OM rooting medium with or without a 5-d dark pre-treatment. Within columns letters indicate statistically different values (Tukey's test, $P \leq 0.05$)

	Rooting shoot [%]	Number of roots [shoot^{-1}]	Mean length [mm]
5-d dark	100a	3.33 ± 0.28b	10.2 ± 0.72a
No dark	93b	4.54 ± 0.35a	10.5 ± 0.83a

number of roots *per* shoot decreased in dark pre-treated shoots with respect to control ones exposed to 16-h photophotoperiod. No differences were found for that regards the mean length of roots. Rooting characteristics of Nebbiara explants appeared very satisfactory also in consideration of the successive critical step of *ex vitro* transplantation (Fig. 1).

In conclusion, the *in vitro* shoot culture technique was successfully applied to cultivar Nebbiara for preserving its germplasm.

Fig. 1. Rooting of Nebbiara shoots.

References

Bajaj, Y.P.S.: Cryopreservation and international exchange of germplasm. - In: Sen, S.K., Giles, K.L. (ed.): Plant Cell Culture in Crop Improvement. Pp. 19-41. Plenum Press, New York 1983.

Bekheet, S.A.: *In vitro* preservation of *Asparagus officinalis*. - Biol. Plant. **43**: 179-183, 2000.

Bekheet, S.A., Taha, H.S., Saker, M.M.: *In vitro* long-term storage of date palm - Biol. Plant. **45**: 121-124, 2002.

Briccoli Bati, C., Fodale, A., Mulè, R, Trombino, T.: Trials to increase *in vitro* rooting of *Olea europaea* L. cuttings. - Acta Hort. **474**: 91-94, 1999.

Gagliardi, R.F., Pacheco, G.P., Valls, J.F.M., Mansur, E.: Germplasm preservation of wild *Arachis* species through culture of shoot apices and axillary buds from *in vitro* plants. - Biol. Plant. **45**: 353-357, 2002.

Grout, B.W.: *In vitro* conservation of germplasm. - In: Bhojwani, S.S. (ed.): Plant Tissue Culture. Application and Limitations. Pp. 394-411. Elsevier, Amsterdam 1990.

Leva, A.R., Muleo, R., Petruccelli, R.: Long-term somatic embryogenesis from immature cotyledons. - J. hort. Sci. **70**: 417-421, 1995.

Martino, L., Cuozzo, L., Brunori, A.: Establishment of meristem tip culture from field-grown olive (*Olea europea* L.) cv. Moraiolo. - Agr. Med. **129**: 193-198, 1999.

Mencuccini, M.: Protoplast culture isolated from different tissues of olive (*Olea europaea* L.) cultivars. - Physiol. Plant. **82**: A14, 1991.

Morini, S., Trinci, M., Zacchini, M.: Effect of different photoperiods on *in vitro* growth of Mr.S. 2/5 plum rootstock. - Plant Cell Tissue Organ Cult. **25**: 141-145, 1991.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio-assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Rugini, E.: *In vitro* propagation of some olive (*Olea europaea* var. *sativa* L.) cultivars with different root-ability, and

medium development using analytical data from developing shoots and embryos. - Scientia Hort. **24**: 123-134, 1984.

Rugini, E., Biasi, R., Muleo, R.: Olive (*Olea europea* var. *sativa*) transformation. - In: Jain, S.M., Minocha, S.C. (ed.): Molecular Biology of Woody Plants. Vol. 2. Pp. 245-279. Kluwer Academic Publishers, Dordrecht 2000.

Rugini, E., Caricato, G.: Somatic embryogenesis and plant recovery from mature tissues of olive cultivars (*Olea europea* L.) "Canino" and "Moraiolo". - Plant Cell Rep. **14**: 257-260, 1995.

Rugini, E., Fedeli, E.: Olive (*Olea europaea* L.) as an oilseed crop. - In: Bajaj, Y.P.S. (ed.): Biotechnology in Agriculture and Forestry. Vol. 10. Pp. 593-641. Springer-Verlag, Berlin 1990.

Rugini, E., Luppino, M., de Agazio, M.: Endogenous polyamine and root morphogenesis variations under different treatments in cuttings and in *in vitro* explants of olive. - Acta Hort. **300**: 225-232, 1992.

Rugini, E., Panelli, G.: Olive (*Olea europaea* L.) biotechnology for short term genetic improvement. - Agro-Food-Industry Hi-Tech **4**: 3-5, 1993.

Wang, Q., Gafny, R., Sahar, N., Sela, I., Mawassi, M., Tanne, E., Perl, A.: Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. - Plant Sci. **162**: 551-558, 2002.

Zacchini, M., Morini, S.: The effect of photoperiod reduction on growth of *Prunus insititia* GF655/2 cultures during multiplication and rooting. - Plant Propag. **7**: 14-16, 1995.