Plant Cell, Tissue and Organ Culture **32**: 283–288, 1993.

In vitro shoot regeneration from olive cultivar tissues

Massimo Mencuccini[1] & Eddo Rugini[2]
[1]*Istituto di Ricerche sulla Olivicoltura-CNR, via Madonna Alta 128, 06100 Perugia, Italy*; [2]*Istituto di Ortofloroarboricoltura, Università della Tuscia, via S. Camillo de Lellis, 01100 Viterbo, Italy*

Received 27 September 1991; accepted in revised form 14 August 1992

Key words: leaf petiole, leaf discs, midribs, *Olea europaea* L.

Abstract

Petioles, leaf discs and midribs of several olive (*Olea europaea* L.) cultivars, collected from potted greenhouse plants, field-grown and *in vitro* shoots, were used to test their morphogenic capacity. Adventitious shoots were induced only in petioles from *in vitro*-grown shoots of cultivars Moraiolo, Dolce Agogia and Halkidikis, grown on Olive Medium (OM) plus 18 μM zeatin within 4 to 5 weeks. Regeneration was achieved, both on Murashige and Skoog (MS) and on modified OM, only in the dark. The highest regeneration was achieved directly from the proximal part of the petioles after 2 to 3 weeks in media containing 5 to 40 μM thidiazuron, or with both 10 μM 2-isopentenyladenine +2.2 μM 6-benzyladenine with or without low auxin concentration (not more than 2.5 μM). A few adventitious shoots were also regenerated from callus when it was shifted from auxin and cytokinin media to cytokinin only medium. The regeneration potential was higher in petioles collected from apical nodes than from basal ones. The adventitious shoots were transferred to solid half-strength MS medium supplemented with 4.5 μM zeatin for further development. Several regenerated shoots were rooted and the plantlets hardened in the greenhouse. No apparent differences regarding morphological aspects were observed among the regenerated plantlets or with those obtained by stimulation of axillary buds.

Abbreviations: BA – 6-benzyladenine, IBA – indole-3-butyric acid, NAA – 1-naphthaleneacetic acid, TDZ – thidiazuron (N-phenyl-N′-1,2,3-thidiazol-5-ylurea), 2iP – 2-isopentenyladenine, MS – Murashige and Skoog medium, 1/2 MS – half strength MS, OM – Olive Medium, BN – Bourgin & Nitsch

Introduction

The genetic improvement of olive (*Olea europaea* L.) using both conventional and non-conventional methods is just at the beginning (Scaramuzzi & Roselli 1986; Rugini 1986; Rugini & Fedeli 1990). Both methods present difficulties due to the long juvenile period, the high heterozygosis of species and because *in vitro* regeneration from tissues or callus has not yet been achieved. Only a few reports are available on olive shoot regeneration and they all concern juvenile tissues, e.g. hypocotyls (Bao et al. 1980) and mature cotyledons (Rugini 1986; Cañas & Benbadis 1988). Somatic embryogenesis was also achieved but only from immature zygotic embryos (Rugini 1988) or from seedling root callus (Rugini 1986). In other woody species, regeneration was usually achieved by using leaf discs (James 1987) or, to a lesser extent, by leaf petioles (Stamp et al. 1990). As described in almost all papers, the shoots usually arise directly from tissues or rarely from callus, because cell regeneration capacity is usually lost after de-differentiation of callus. In olive, preliminary results on *in vitro* regeneration of cultivar tissues have been reported by Mencuccini & Corona (1990), showing a potential capacity of this

species to regenerate. This paper reports on several attempts of shoot regeneration in olive cultivars, in particular shoot regeneration and plant recovery from olive leaf petioles.

Materials and methods

Plant material

The explants used for these experiments were collected from:
- 1-year-old greenhouse-grown potted plants, cv Cipressino;
- 10-year-old field-grown plants of cv Moraiolo and Dolce Agogia;
- *in vitro*-cultured shoots of the cv Moraiolo, Dolce Agogia and Halkidikis.

Petioles, leaf discs and midribs were used to test the morphogenic capacity. Explants from greenhouse and field-grown plants were obtained by surface disinfesting the single nodes for 20 min in a solution containing 6% sodium hypochlorite plus 3 drops of Tween 80 per 100 ml, then rinsing three times in sterile distilled water.

In all experiments the explants were collected from *in vitro* growing shoots that had already been established *in vitro* 5 to 6 years before on OM medium, with 40-day subcultures of one-node explants under environmental conditions reported previously (Rugini 1984). The leaf petioles were isolated from actively growing 28-day-old cultures with great care to exclude adjacent axillary buds, and placed on the medium in an horizontally oriented position in order to determine the origin of shoots. Leaf discs were made with a 7 mm borer in the middle of the lamina, while the midribs were isolated by two longitudinal and parallel knife cuts leaving only 1–2 mm of leaf blade on both sides. The midribs were tested because it is known that shoots often arise near them. This was attempted to avoid the adjacent non-morphogenic tissues from inhibiting morphogenesis. Both kinds of explants were placed with the abaxial surface in contact with the medium. In an attempt to compare regeneration capacity of explants in different positions along the shoot, one experiment was carried out by using petioles collected separately from the last three apical and three basal nodes. In all the other experiments, all the explants collected from whole shoots were randomly mixed.

Media environmental conditions and treatments

Three basal media for regeneration experiments were used: 1) half-strength MS (Murashige & Skoog 1962), 2) full-strength MS and 3) OM (Rugini 1984). The latter was modified by replacing the macroelements with those of BN (Bourgin & Nitsch 1967) and supplemented with $1\ g\ l^{-1}$ casein acid hydrolysate. All the basal media were supplemented with 2% sucrose. The pH was adjusted to 5.5 before adding 0.7% agar (Difco Bacto) and autoclaved at 121 °C for 15 min. Each treatment included 5 petri dishes (25×90 mm) with 25 ml medium, containing 8 explants, for a total 40 explants for each treatment. The cultures were placed in a growth room at 23 ± 1°C, and according to the experiments, in the dark or in a 16-h photoperiod, under cool white fluorescent lamps at 40 μmol $m^{-2}\ s^{-1}$. All data were subjected to an analysis of variance. This experimental scheme, as well as the environmental conditions, were used for all the experiments. The regenerated shoots were rooted singly in 120×12 mm test tubes with aluminium screw caps, containing 5 ml medium. The medium contained half-strength BN macroelements, BN microelements and vitamins, 4 μM NAA and 2% sucrose. The cultures were rooted under 16-h photoperiod. The plantlets were then transferred to pots containing peat moss and perlite (1:1, v/v) for acclimatization.

Experiment 1

In this experiments the petioles and leaf discs of 1-year-old greenhouse-grown and 10-year-old field-grown plants were tested for their morphogenic capacity. Field-grown plant petioles, of cultivars Moraiolo and Dolce Agogia, collected 30 days after full-bloom, were tested in an MS basal medium supplemented with factorial combinations between NAA (0, 1.25 or 5.0 μM) with TDZ (0, 1.0, 5.0 or 10.0 μM), and in a modified OM medium plus 10 μM 2iP and 2.2 μM BA with or without 2.5 μM IBA.

Greenhouse potted-plant petioles, from cv

Cipressino, collected in April during active growing, were tested on the modified OM medium and growth regulator combination. The petioles collected from the three apical nodes of the lateral shoots were maintained separated from those of the three basal ones. The cultures were grown either in the dark or in 16-h photoperiod.

Experiment 2

Leaf discs and petioles from *in vitro*-grown cv Moraiolo were tested, both in half-strength and full-strength MS medium, both supplemented with the factorial combinations between TDZ (0, 0.1, 1.0, 5.0, 10.0 or 20.0 μM) and NAA (0, 2.5, 5.0, 10.0 or 20.0 μM). The cultures were either grown in 16-h photoperiod or in the dark.

Experiment 3

According to the results obtained with the previous experiment, leaf petioles of 3 cultivars (Moraiolo, Dolce Agogia and Halkidikis) were tested only in MS full-strength medium supplemented with factorial combinations of growth regulators between NAA (0, 1.25, 2.5 or 5.0 μM) with TDZ (0, 5.0, 10.0, 20.0, 40.0 or 80.0 μM) or with the same concentration of NAA as above with BA (0, 2.5, 5.0 or 10.0 μM). The cultures were only grown in the dark.

Experiment 4

This experiment on modified OM medium used leaf discs, petioles and midribs to evaluate the results obtained by using the *in vitro*-grown cultivar Moraiolo. Two growth regulator combinations: 10 μM 2iP + 2.2 μM BA and 10 μM 2iP + 2.2 μM BA + 2.5 μM IBA were tested. In this experiment, the petioles collected from the last three apical nodes and those from three basal ones were kept separately. All cultures were grown in the dark.

Results

Explants from both field and greenhouse plants did not produce adventitious shoots. The petioles produced only a large amount of hard callus in modified OM medium containing both cytokinin and auxin. The callus transferred to auxin-free medium continued to grown for only 5–10 days, then became brown. In auxin-free medium with only cytokinin, they soon became brown both in the light and in the dark. The leaf discs rarely produced callus adjacent to the main veins.

From *in vitro*-cultured olive shoots in all media and conditions tested, the leaf discs and the midribs did not regenerate any shoots. They

Table 1. Average shoot regeneration in darkness from leaf petioles and callus formation of three cultivars established *in vitro*.

Cultivars	Growth regulators (μM)	Basal media					
		1/2MS		MS		Modified OM	
		Regeneration (%)	Callus formation	Regeneration (%)	Callus formation	Regeneration (%)	Callus formation
Moraiolo	Thidiazuron 5.0	0.0*	0/+	18.7 ± 1.0	0/+	–	–
	Thidiazuron 10.0	0.0	0/+	11.2 ± 1.0	0/+	–	–
	Thidiazuron 20.0	8.7 ± 0.7	0/+	10.0 ± 0.9	0/+	–	–
	Thidiazuron 40.0	–	–	8.7 ± 0.9	0/+	–	–
	Thidiazuron 10.0 + NAA 2.5	0.0	++	10.0 ± 0.6	++	–	–
	2iP 10.0 + BA 2.2	–	–	–	–	17.5 ± 0.7	0/+
	2iP 10.0 + BA 2.2 + IBA 2.5	–	–	–	–	3.7 ± 0.6	++
Dolce Agogia	Thidiazuron 10.0	–	–	5.0 ± 0.7	0/+	–	–
Halkidikis	Thidiazuron 10.0 + NAA 1.25	–	–	5.0 ± 0.9	+	–	–

Average of petioles collected from whole shoots of 6 to 7 nodes. Only the growth regulator combinations that gave regeneration is reported. (0 = no callus; + = small callus; ++ = large callus; – = no data)

*Numbers represent the mean ± SE of two experiments of 5 replications each with 40 explants per treatment per experiment. Each explant invariably gave only one shoot.

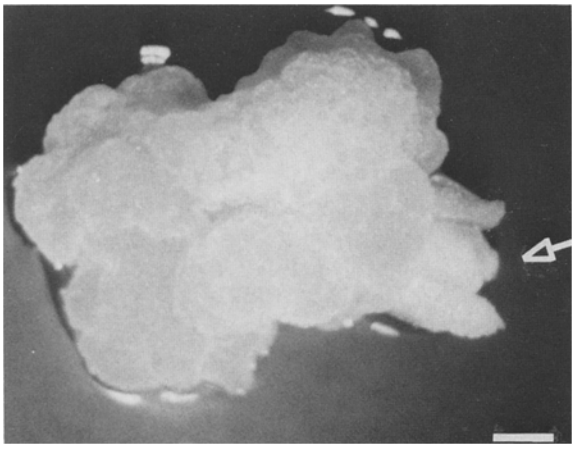

Fig. 1. Regeneration from leaf petiole callus on medium containing TDZ 10 μM and NAA 2.5 μM (cv Moraiolo). (bar = 0.12 mm)

produced callus around the cut surface only in media containing both cytokinins and auxins; in absence of auxin the explants became brown before starting to produce callus or after a small callus formation.

In contrast, the petioles were able to regenerate shoots. Table 1, in which only the growth regulator combinations that induced differentiation have been reported, shows that the percentage of explants forming shoots was strongly dependent on the cultivar, the cytokinin and auxin concentration, and the light conditions. The cultivar Moraiolo showed a greater regeneration potential than cv Dolce Agogia and cv Halkidikis. In the light, regeneration was never observed and auxin was essential to induce callus production, which was hard and green.

In the dark, all petioles, within 5–7 days of culture on media containing both cytokinins and auxin, underwent a slight swelling in the distal position and started to become somewhat white. By two weeks, all explants that had survived the initial culture period (more than 85%) showed callus formation. Only 2–5% of these explants regenerated shoots but only when the auxin concentration was not more than 2.5 μM (Fig. 1).

In all basal media tested containing only cytokinins (TDZ, 2iP + BA), only 30–40% of the explants survived. By 15–20 days, adventitious structures started to show their proximal end in a range of 5–40 μM concentration. After 3 weeks only one shoot from each explant was visible, in presence or in absence of callus (Fig.

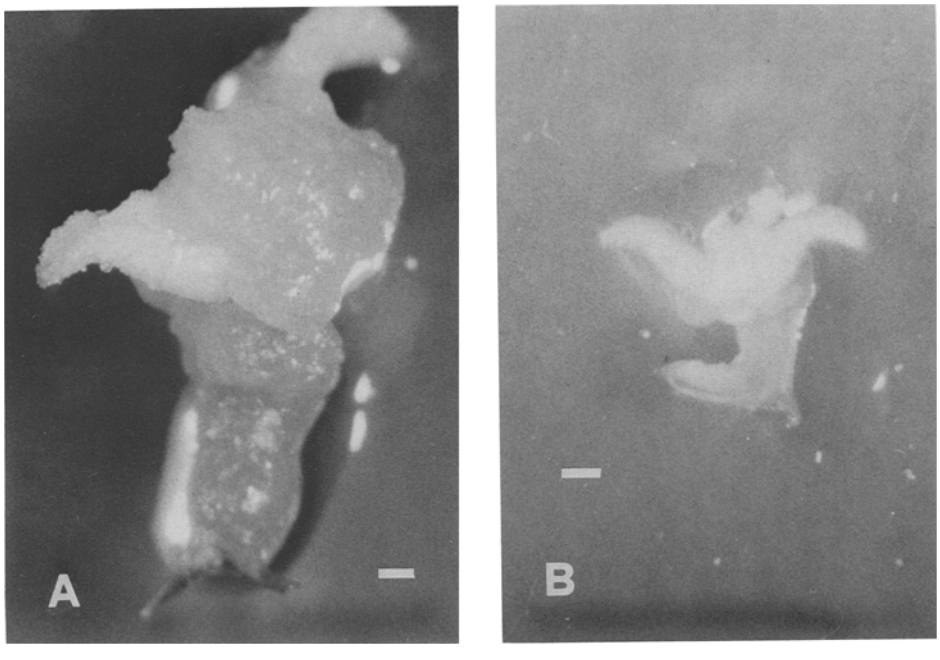

Fig. 2. Regeneration from leaf petiole in presence (*A*) and in absence (*B*) of callus, on auxin-free medium containing TDZ 5 μM (cv Moraiolo). (bar = 0.3 mm in *A*, 0.6 mm in *B*)

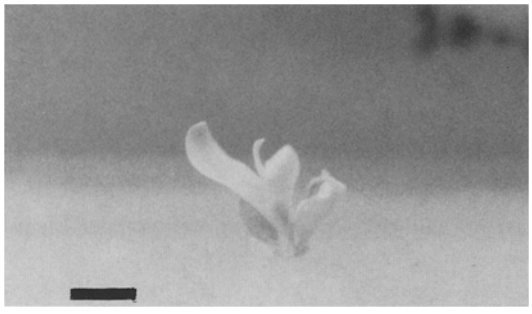

Fig. 3. Shoot regenerated from leaf petiole on growth medium (cv Moraiolo). (bar = 6.2 mm)

2). The regeneration was achieved in both MS or modified OM media, while the half-strength MS seemed to be less efficient (Table 1). In media containing only BA, with or without auxin, no shoot regeneration occurred (data not shown).

The regeneration response varied considerably with the position of the petiole along the stem. Petioles obtained from the three apical nodes regenerated in a higher percentage ($40 \pm 2\%$) than the basal ones ($5 \pm 1\%$) on OM modified medium with 10 μM 2iP and 2.2 μM BA. When 2.5 μM IBA was added to this medium, only petioles from apical nodes regenerated shoots

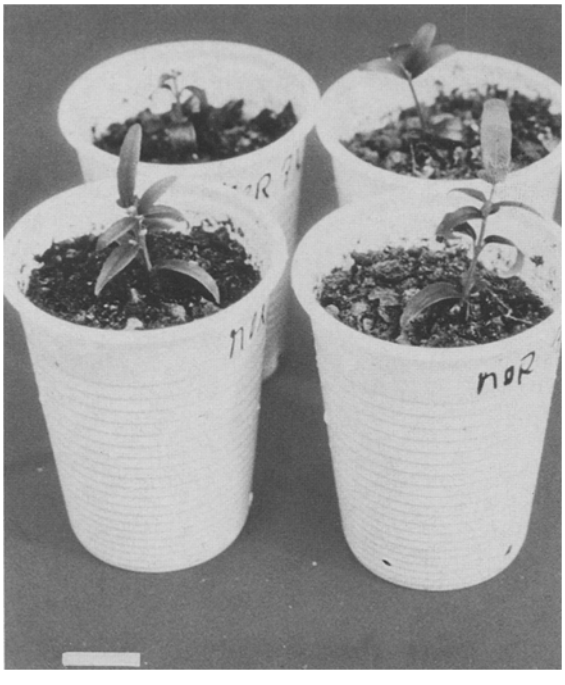

Fig. 4. Plantlets regenerated from leaf petiole, established in soil in the greenhouse (cv Moraiolo). (bar = 2.7 cm)

($2 \pm 1\%$). However, the number of shoots per explant was invariably one.

Two weeks after regenerated shoots appeared, having produced 2–3 nodes, they were transferred to the solid half-strength MS medium supplemented with 4.5 μM zeatin under a 16-h photoperiod for further development (Fig. 3). They contained to grow, showing the same behaviour as their mother plants, as far as proliferation rate, rooting ability and soil adaptation were concerned. The hardened plants, both those regenerated directly from petiole tissue and those regenerated from callus, showed an identical morphology (Fig. 4).

Discussion

This study clearly shows that shoot regeneration occurs only from *in vitro* growing shoots. Attempts to regenerate adventitious shoots from leaf discs and petioles from rapidly growing field or young greenhouse potted plants of the cultivars tested were unsuccessful. At present, this regeneration technique is dependent on the possibility of establishing *in vitro* olive cultivars, which, notwithstanding many attempts, is still quite difficult for several cultivars (Rugini & Fedeli 1990). In addition, regeneration efficiency seems to be quite low, since it was only possible to recover one shoot per explant. However, we think that the percentage of petioles forming adventitious shoots could be increased by improving the *in vitro* shoot proliferation and environmental growth conditions. Regeneration from callus is still difficult; however, it was possible to recover some shoots by switching the petiole callus from the auxin and cytokinin media to auxin-free-medium (data not shown).

A different response in regeneration capacity among the cultivars has also been observed. 'Moraiolo' was more responsive than the other two, 'Dolce Agogia' and 'Halkidikis'. We are sure that this difference should only be ascribed to the different genotype, because the *in vitro* cultures were initiated at the same time from the same mature buds and maintained in the same environmental and medium conditions. Since the petioles from *in vitro* growing shoots are morphogenically competent, as tissues from seed-

lings (Cañas & Benbadis 1988), contrary to those from field and greenhouse explants, it seems that mature cultivars need a period *in vitro* in order to be rejuvenated.

Among several variables that could modify the morphogenic response, darkness was essential for successful regeneration, as was already observed for somatic embryogenesis from immature zygotic embryos of olive (Rugini 1988). In our experience up to now, even when light of different wavelength was applied during the shoot proliferation phase, no subsequent change in the regeneration capacity of petioles, placed in the dark, was observed; when light of different wave length was applied during the regeneration phase, the petioles did not show any regeneration capacity as was found also with those placed under white light (unpublished data).

Acknowledgements

This work was supported by a grant from the Italian Ministry of Agriculture and Forestry, project 'Tecnologie Avanzate Applicate alle Piante' and National Research Council of Italy, Special Project RAISA, Subproject No. 2, Paper 545.

References

Bao Z-H, Ma Y-F, Liu J-F, Wang K-J, Zhang P-F, Ni D-X & Yang W-Q (1980) Induction of plantlets from the hypocotyl of *Olea europaea* L. *in vitro*. Acta Bot. Sin. 2: 96–97 (in Chinese)

Bourgin J P & Nitsch J P (1967) Obtention de *Nicotiana* haploides à partir d'ètamines cultivèes *in vitro*. Ann. Physiol. Veg. 9: 377–382

Cañas L A & Benbadis A (1988) *In vitro* plant regeneration fromn cotyledon fragments of the olive tree (*Olea europaea* L.) Plant Sci. 54: 65–74

James D J (1987) Cell and tissue culture technology for the genetic manipulation of temperate fruit trees. Biotechnol. Genet. Eng. Rev. 5: 33–79

Mencuccini M & Corona C (1990) Genetic improvement of olive by *in vitro* culture. Proc. XXIII Int. Hort. Cong. Firenze, Aug. 27-Sept. 1, (p. 3126) (abstract)

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Rugini E (1984) *In vitro* propagation of some olive (*Olea europaea* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Scientia Hort. 24: 123–134

Rugini E (1986) Olive. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry Trees, Vol 1 (pp 253–267). Springer-Verlag, Berlin, Heidelberg, New York, Tokyo

Rugini E (1988) Somatic embryogenesis and plant regeneration in Olive (*Olea europaea* L.). Plant Cell Tiss. Org. Cult. 14: 207–214

Rugini E & Fedeli E (1990) Olive (*Olea europaea* L.) as an oilseed crop. In: Bajaj YPS (Ed) Biotechnology in Agriculture and Forestry. Legumes and Oilseed I. (pp 593–641). Springer-Verlag, Berlin Heidelberg, New York, Tokyo

Scaramuzzi F & Roselli G (1986) Olive genetic improvement. Olea 17: 1–17

Stamp J A, Colby S M & Meredith C P (1990) Improved shoot organogenesis from leaves of grape. J. Amer. Soc. Hort. Sci. 115: 1038–1042