Involvement of Polyamides in Auxin and Agrobacterium rhizogenes - induced Rooting of Fruit Trees in Vitr

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Additional index words. micropagation, transformation, olive, apple, almond, pistachio

Abstract. In the olive Olea europaea (L.), the polyamides (PAs), putrescine, spermidine, and spermine, when added exogenously at a concentration of 1 mM in the in vitro rooting medium, combined with 5 mM auxin concentration, promoted early rooting and increased the final rooting percentage and the number of roots per explant. The effect was less evident in olive explants rooted with the basal blanching method; thin-layer chromatography of total endogenous PAs in these explants revealed lower levels on day 2 compared with controls, while by day 5 PA concentrations in both had fallen to similar levels. Furthermore, putrescine decreased the pH of the medium by 0.5 units around the explants. PAs had little effect on apple Malus pumila (Mill.) and no effect on almond Prunus dulcis (Miller) D.A. Webb and pistachio Pistacia vera (L.). There were also some positive effects observed, but only in olive, when rooting was induced by Agrobacterium rhizogenes in auxin-free medium. Few plantlets showed agropine-positive roots.

Materials and Methods

Experimental plant tissue included olive shoots of cultivars Moraio and Dolce Agogia, which have characterized varable rooting efficiency, apple (Malus pumila Mill.) of unknown genotype, Fiasconello almond, a gamma-ray mutation, and pistachio seedlings. Olive shoots had been maintained for 4 years in vitro (with subculturing every 35 to 40 days) in OM (olive basal medium, plus 18 μM zeatin riboside, 3% sucrose, and 0.7% agar) (Rugini, 1986b). Apple shoots also had been maintained in culture for 4 years on Linsmaier and Skoog (1965) medium with 4.4 μM BAP and 0.5 μM IBA, according to James and Turbon (1979). Pistachio and almond had been maintained in culture for 2 years (with subcultures at 30 and 21 days, respectively) on Murashige and Skoog (1962) (MS) medium plus 1.5 μM BAP, 0.05 μM NAA, 3% sucrose, and 0.8% agar (Rugini, 1986a). All cultures were maintained under a 16-h photoperiod at 40 μmol·m⁻²·s⁻¹ provided by cool-white fluorescent lamps at 24 ± 1°C. Half-strength MS salts and vitamins supplemented with 2% sucrose, 0.8% w/v Difco Bactoagar and either 0.5 or 5 μM NAA, at pH 5.5 were used as a rooting medium. All media were autoclave for 15 min at 121 C. The polyamides putrescine (Put), spermidine (Spd), and spermine (Spm) were filter-sterilized and added to the rooting medium at 0.7% agar (Rugini and Wang, 1986). Most of the experiments were carried out using diamine putrescine.

One experiment compared the effect of putrescine on rooting by exposing the explants to a 16 h-light photoperiod at 60 μmol·m⁻²·s⁻¹ with or without basal blanching (Rugini et al., 1987). The technique consisted of maintaining the basal part of shoots in darkness during the entire period of rooting by blackening the outside of the jars up to 3 to 4 mm above the medium and by covering it with small black polycarbonate granules.

Experiments with A. rhizogenes (strain NCPPB 1855) were carried out using the same basal rooting medium without auxins. In addition, 5-azacytidine at 28 μM, auxins [1H-indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthalene-acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D)], zeatin, and gibberellic acid (GA₃) at 5 μM (Table 3) were tested in an attempt to increase bacterium-induced rooting efficiency. Bat-
ttrial inoculation was carried out by making two superficial longitudinal cuts (5 to 7 mm long) with an infected knife on the basal portion of the explants. This procedure was compared with a bacterial inoculation by puncturing the middle of the explants with a glass needle.

The rooting tests with auxin or with *A. rhizogenes* involved 60 in vitro-grown shoots per treatment, with two to three nodes for olive and five to six for the other species. The explants were placed separately in test tubes (120 × 16 mm) containing 5 ml of medium. The cultures were then placed in a growth chamber at 24 ± 1C under 40 µmol·m⁻²·s⁻¹ light intensity, supplied by cool-white fluorescent lamps; the photoperiod was 16 h. Root growth was recorded every 7 to 10 days, according to the experiments and the species examined. At the end of the experiment, the explants were extracted from the test tube and a pH sensor was placed where the explants had been, to measure the pH of the medium. The number of roots per explant was also recorded. The roots of 15 randomly chosen explants rooted by *A. rhizogenes* were analyzed for opines by paper electrophoresis (White et al., 1982). Data were analyzed by the x² test. Plantlets were then transferred to a mixture of 1.5 peat moss : 1.0 perlite : 0.5 polystyrene granules (by volume) in boxes covered with polyethylene film and placed under a 16-h photoperiod at 20 to 70 µmol·m⁻²·s⁻¹ in a growth room held at 20 ± 1C. Before being transferred into pots, the plantlets rooted with *A. rhizogenes* were washed for 24 h in a shaker that contained vancomycin solution at 200 mg-liter⁻¹.

**Polyamine determination.** Perchloric acid (PCA) and total polyamine (Put, Spd, and Spin) concentrations were determined by thin layer chromatography (TLC) (Torregiani et al., 1990) in chloroform : ethylacetate (4:2) from a 500-mg fresh weight sample. The shoots used for total PA determination were first divided into apical and basal parts, then cultured in rooting medium under the same environmental conditions as those used for the rooting test, with or without (control) basal blanching. The analyses were carried out at 2 and 5 days after the beginning of rooting, a crucial period for the root induction phase, by homogenizing duplicate samples of the whole explants.

**Results**

**Olive.** Both cultivars, Moraiole and Dolce Agogia, gave similar rooting responses under auxin and PA treatments. PAs affected rooting only in combination with auxin treatment, increasing rooting percentage and promoting earlier root emergence compared with the explants placed on medium with auxin only; Put seemed to be a more active PA than the other two (Fig. 1). Further, Put + NAA also increased the number of roots per explant (Table 1). In auxin-free medium, with or without PAs, not more than 8% of the explants had rooted (data not shown). The aim of the experiment was to examine the effect of Put at two auxin concentrations. There was a higher percentage of rooted explants in medium containing 5 µM NAA than in medium containing 0.5 µM NAA, but when 1 mM Put was added to the media there was no difference in the final rooting percentage (Fig. 2), although the explants in the medium with lower auxin concentration rooted more slowly. At the lower auxin concentration, with or without Put, explants rooted without any basal callus formation, contrary to those placed in 5 µM NAA, which formed a large basal callus; the addition of Put promoted further growth (data not shown). When rooting was carried out with basal blanching, Put was not as effective as in the blanched explants, since this method dramatically increased the rooting percentage (Fig. 3).

![Fig. 1. Effect of 1 mm polyamines (putrescine (Put), spermidine (Spd), and spermine (Spm)) on rooting of ‘Moraiole’ olive explants on medium containing 5 µM NAA. Triangles indicate the difference by x² test of homogeneity at P = 0.05 with the corresponding control mean. Put (○—○); Spd (△—△); Spm (□—□); Control (●—●). In auxin-free medium, rooting was absent or minimal. Each datum is the mean of 60 explants.](image)

**Table 1. Root formation per explant ‘Moraiole’ olive after 42 days on half-strength MS rooting medium with various supplements. NAA and putrescine (Put) were added to the medium at 5 µM and 1 mM, respectively.**

| Medium supplement | No. explants with no. roots indicated | Total |
|-------------------|--------------------------------------|-------|
| None              | 0 1 2 3+ Total                       |       |
| Put               | 55 3 41 0 a’ 60                      |       |
| NAA               | 33 11 12 4 b 60                      |       |
| NAA + Put         | 11 8 18 22 c 59                      |       |

Total endogenous PAs were present at a lower concentration in shoots derived from the basal blanching method than the control at 2 days. By 5 days all PAs had decreased in concentration, and it was the same in the explants from the two rooting methods (Table 2).

At the end of the experiment, the medium containing 5 µM NAA had a pH of 4.9 ± 0.06 SD, whereas in that containing NAA and Put, the pH was significantly lower (4.4 ± 0.08 SD) around the basal portion of the explants. The variation was similar in media containing rooted and that containing unrooted explants.

Treatment with *A. rhizogenes* alone promoted rooting in 26 out of 58 treated explants. An auxin concentration of 5 µM in the medium did not increase the rooting percentage when rooting was induced by the bacteria; 2,4-D inhibited rooting and promoted a large basal callus. Zeatin and GA, at the same auxin concentration, decreased rooting drastically (Table 3); 5-azacytidine at 28 µM delayed only the early stage of root initiation (data not shown). Putrescine promoted early rooting and increased the final rooting percentage (Fig. 4, Table 3), as already observed in explants rooted by auxin. The roots emerged from

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J. Amer. Soc. Hort. Sci. 117(3):532-536. 1992. 533
the basal portion of explants in most cases even when inoculation was done with a glass needle in the middle of the explant. Rooting efficiency was similar between the two inoculation methods tested.

**Other species.** In almond and pistachio, putrescine had no effect on rooting, in apple it had a minimal effect, since it promoted slightly earlier rooting and increased the rooting percentage when compared with shoots rooted with auxin alone (data not shown). When explants were transformed with *A. rhizogenes*, putrescine increased bacterial growth around the explants, particularly in almond and pistachio, but not in apple and olive. In all these species, as in olive, Put clearly increased basal callus formation (data not shown). Also, the roots in most cases emerged at the base of the explant, as observed in olive. In all species, only 20% of the plantlets that rooted with *A. rhizogenes* showed agropine-positive roots, including those grown in medium containing the hypomethylant drug 5-azacytidine.

Plant survival in pots was usually high, ≈90%. In some potted olive and almond plantlets, observed after 6 months in pots, the root system induced by *A. rhizogenes* clearly had more secondary roots than shoots rooted with auxins. However, the root systems were similar after 2 years in pots.

**Discussion and Conclusion**

The present work shows that polyamides promote early rooting and increase rooting percentage in olive explants treated with auxin or with *A. rhizogenes*. The polyamine effect, however, was partially masked by rooting the explants by basal blanching. In shoots that were rooted by this method, the total endogenous PAs were present in low concentration at 2 days. Darkness probably promoted rapid PA degradation, and the catabolic products, e.g., hydrogen peroxide, might contribute to maintaining a low concentration of IAA during the induction phase. Putrescine treatment lowered the pH of the medium around the explants, and this decrease could affect rooting. This fact makes it even more difficult to understand how PAs interfere with rooting, directly or indirectly even with their catabolic products. However, it is clear that their presence in the tissue is essential for rooting. The inhibition of polyamine synthesis by dymethylfluoronithine (DMFO) and dymethylfluroarginine (DMFA) in *Hedera helix* L. (Geneve, 1987) and in cherry (Biondi et al., 1990) resulted in a low frequency of root primordia, supporting the hypothesis of Friedman et al. (1982) who suggested a possible regulatory role for PAs in combination with auxins in the early phase of adventitious root formation. Since Spd has been found to increase phenolic compounds in rose cell suspensions (Muhitch and Fletcher, 1986), it might contribute...
Table 3. Rooting of ‘Moraiolo’ olive explants with A. rhizogenes at 70 days in combination with growth regulators (5 µM), 5-azacytidine (28 µM) or putrescine (1 mM).

| Growth regulator | Explants (no.) | Rooted (%) | Unrooted (%) | Total (%) |
|------------------|----------------|------------|--------------|----------|
| Control          |                |            |              |          |
| IAA              | 26             | 32         | 58           |
| IBA              | 29             | 31         | 60           |
| NAA              | 30             | 30         | 60           |
| 2,4-D            | 26             | 34         | 60           |
| Zeatin           | 11             | 48         | 59           |
| GA₃              | 3              | 57         | 60           |
| 5-azacytidine    | 22             | 38         | 60           |
| Putrescine       | 41             | 18         | 59           |

Separation between untreated control and each treatment by x² test of homogeneity, P = 0.05.

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