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Plantlets from encapsulated micropropagated buds of M.26 apple rootstock

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Abstract

In order to be considered usable as synthetic seeds, encapsulated explants sown under *in vitro* or *ex vitro* conditions must be able to produce whole plantlets. Ninety percent of non-encapsulated M.26 apple rootstock single nodes produced a plantlet (i.e., a well-formed shoot with a root system) after 30 days of culture *in vitro* if the explants were previously given a 24-hour treatment with 24.6 μM IBA and 15 g l^{-1} sucrose in darkness. In contrast, when the single nodes were encapsulated in a calcium-sodium alginate bead immediately after the same treatment only 10% of the encapsulated explants formed a plantlet. Addition of growth regulators to the artificial endosperm and culture of the single nodes for root primordia initiation for 3, 6 or 9 days in darkness before encapsulation allowed production of 58%, 60% and 66% of plantlets, respectively.

Abbreviations: $AdSO_4$ – adenine hemisulfate; BA – benzyladenine; GA_3 – gibberellic acid; IBA – indolebutyric acid; PVP – polyvinyl pyrrolidone; Synseed – synthetic seed

Introduction

Synthetic seeds (*synseeds*) have been recently defined as artificially encapsulated somatic embryos, shoots or other tissues, that can be used for sowing under *in vitro* or *ex vitro* conditions (Aitken-Christie et al., 1995). In previous definitions, the only explant type that was considered for synseed production was the somatic embryo, which is a bipolar structure (Redenbaugh, 1993). Somatic embryogenesis, though, has not been achieved for all species, and micropropagated buds (unipolar explants) offer an interesting alternative (Bapat, 1993, Piccioni and Standardi, 1995). In fruit trees such as apple, which is studied worldwide for breeding and propagation purposes, encapsulated unipolar explants could be useful in exchanges of sterile material among laboratories, because of the small size and relative ease in handling these structures, and also in plant propagation and nurseries, if the development of the plant could be properly directed toward proliferation, rooting, elongation, etc. (Bapat, 1993; Piccioni and Standardi, 1995).

In mulberry and banana, plantlets were obtained from alginate-encapsulated buds without any specific root induction treatment (Bapat and Rao, 1990; Ganapathi et al., 1992). Previous experiments with encapsulated buds of apple rootstocks and cultivars demonstrated that shoots, but not roots, could be regenerated after *in vitro* sowing, even after medium-term cold storage (Piccioni et al., 1992; Piccioni and Standardi, 1995; Piccioni et al., 1996). In order to achieve rooting in micropropagated apple shoots, auxin and dark treatments are generally necessary (Zimmerman, 1984). Welander and Pawlicki (1993) calculated that the optimal auxin concentration to obtain rooting of micropropagated stem disks of apple was 24.6 μM IBA applied in an agar-gelled medium for 24 hours in darkness.

The objective of this study was to obtain both rooting and shoot elongation (i.e., plantlets) from encapsulated micropropagated buds of the clonal apple rootstock M.26 after *in vitro* sowing of the alginate beads.

Table 1. Composition of the medium used in the experiments.

Component	Concentration	Component	Concentration
INORGANICS		ORGANICS (μM)	
Macroelements (mM)		Nicotinic acid	8.10
NH_4NO_3	5.0	Thiamine-HCl	3.00
KNO_3	17.8	Myo-inositol	560.00
$MgSO_47H_2O$	1.5	Biotin	0.40
KU_2PO_4	2.0	Folic acid	0.02
$Ca(NO_3)_24H_2O$	5.0	Ca-panthotenate	1.00
		Riboflavin	0.26
Microelements (μM)		p-aminobenzoic acid	7.30
KI	0.5		
H_3BO_3	194.0		
$MnSO_4H_2O$	4.4	GROWTH REGULATORS (μM)	
$ZnSO_47H_2O$	30.0		
$Na_2MoO_42H_2O$	1.0	IBA	0.5
$CuSO_45H_2O$	0.1	GA_3	1.4
$CoCl_26H_2O$	0.1	BA	2.2
Na_2EDTA	100.0	$AdSO_4$	2.5
$FeSO_47H_2O$	100.0		
SUCROSE (mM)	87.6	AGAR (g l^{-1})	7.0
pH	5.5		

Materials and methods

Stabilized shoot cultures of the clonal apple rootstock M.26 (*Malus pumila* Mill.) were obtained following a modified version of the protocol by Jacoboni and Standardi (1982). Briefly, dormant 1-year-old shoots with 2-3 buds were selected from field mother plants during the winter, after completion of chilling requirements. The shoots were surface disinfested for 30 minutes with 9% calcium hypochlorite, rinsed three times with sterile distilled water and then stored in a 0.1% PVP sterile solution. The scales were removed from each bud under aseptic conditions in order to expose the meristematic tissues, then each meristem with the first two leaf primordia was excised and transferred to a test tube with a 0.7% w/v agar-gelled (Sigma, A-1296) medium, identical to the proliferation medium reported in Table 1, modified by reducing the concentrations of macro- and micro-nutrients and organics to one half and of the growth regulators to one tenth. The pH of the medium was adjusted to 5.5 before addition of sucrose and agar. Vessels and media were autoclaved at 110°C for 20 minutes. At the end of the first month, the uncontaminated shoots were harvested, subdivided into 1-2 cm portions containing at least 2-3 buds and transferred to fresh medium. After 3 monthly subcultures in the same conditions, the proliferated shoots were subcultured in 100 ml full strength medium (Table 1) in 500-ml glass vessels, 15 shoots/vessel, and maintained in a growth chamber at 22±2°C, under a 16 hour light photoperiod and a 40 μmol $m^{-2}s^{-1}$ photon flux, provided by 30-watt cool-white neon lamps. At the end of each monthly subculture period, healthy green shoots longer than 1 cm were excised and transferred to fresh medium for further proliferation.

After several proliferation subcultures, shoots randomly chosen at the end of a monthly subculture period were used to obtain apical and axillary buds for encapsulation. The shoot axis was cut 1-2 mm above and below each node, and 5-mm-long single-node cuttings were obtained. No distinction between axillary and apical buds was ever made throughout the experiments, as all explants were randomly mixed and used as experimental units. The single nodes were transferred immediately after excision into 50-ml closed glass vessels (10 explants/vessel) containing 15 ml of a

liquid solution with 24.6 μM IBA and 15 g l^{-1} sucrose, pH adjusted to 5.5. The vessels were incubated in the growth chamber on a 100 rpm rotary shaker for 24 hours in darkness. This 24- hour step will be referred to as the auxin treatment.

Experiment 1

The aim of this experiment was to verify the ability of the single nodes coming from the auxin treatment to form plantlets, with or without sodium alginate encapsulation. At the end of the auxin treatment, the single nodes were divided into two groups. The first group was not encapsulated. The single nodes from the second group were encapsulated in alginate beads as previously reported (Piccioni and Standardi, 1995). In order to insert an artificial endosperm into the beads, a nutritive solution made of the the proliferation medium (Table 1) at half strength, without growth regulators and without agar was prepared and then divided in three fractions. The first fraction was used to prepare the encapsulation matrix, adding 2.5% w/v sodium alginate (Sigma, A-2033) to the solution. The second fraction was used to make the complexing mixture by adding $CaCl_2$ to the solution to obtain a final concentration of 100 mM. The third fraction was used without further additions for the final rinse. All mixtures were adjusted to pH 5.5 and then autoclaved at least 1 day before use. For encapsulation, the nodes were immersed for a few seconds with a forceps into the encapsulation matrix, in order to allow them to be covered by the gel. The alginate-covered nodes were then placed in the $CaCl_2$ solution while stirring for 30 minutes. The hardened alginate capsules containing the nodes were then rinsed twice for 10 minutes each with the rinse solution and then used for sowing.

Naked and encapsulated nodes were placed in separate Magenta vessels (7×7×7cm) containing 50 ml of a regrowth medium, made of half strength proliferation medium (Table 1) without growth regulators, solidified with 7 g l^{-1} agar. Ten randomly chosen single nodes were cultured in each vessel. Each vessel was considered as one replicate in a randomized block experimental design. Viability and regrowth values were monitored after one month in the growth chamber. The nodes were considered alive if they had maintained a green appearance, with no necrosis or yellowing. Regrowth was evaluated as an increase in size, with extrusion of a shoot and a green leaf or of a root. Regrown nodes were also monitored for sprouting and rooting rates. Only the percentage of shoots longer than 5 mm was considered for sprouting rates. The whole experiment was conducted twice.

Experiment 2

The aim of the second experiment was to find the optimal interval after the auxin treatment for encapsulation of the single nodes, in order to allow the highest plantlet formation rate after sowing of the capsules. Therefore, the single nodes from the auxin treatment were not encapsulated immediately. Instead, they were first allowed to initiate root primordia, which in apple micropropagated explants occurs in the first 3-9 days after induction (Welander and Pawlicki, 1993). For root primordia initiation, ten single nodes coming from the auxin treatment were placed in each Magenta vessel on 50 ml of regrowth medium, identical to that of Experiment 1. In order to enhance gas exchange between the explants and the environment, the nodes were not immersed in the medium, but instead placed on a sterile paper filter (Whatman # 1), previously laid flat on the medium surface. The vessels were divided into 3 lots and kept in darkness in the growth chamber for 3 days, 6 days or 9 days. At the end of each root primordia initiation culture period, visual observations were conducted on the nodes to monitor possible early rooting and/or sprouting. Callus production on the explants was also visually detected. After these observations, no sorting of the nodes was made, and all were encapsulated in sodium alginate, following the same procedure adopted in Experiment 1. The artificial endosperm used was identical to that described for Experiment 1, modified by adding 0.25 μM IBA, 1.11 μM BA, 0.72 μM GA_3 and 1.24 μM $AdSO_4$ to the nutritive formulation. After encapsulation, the capsules were sown in the regrowth medium. All regrowth conditions were as described for Experiment 1. Each regrowth vessel with 10 encapsulated single nodes was considered as one replicate of a randomized block experimental design. Five replicates (i.e., 50 single nodes) were used per each treatment. After 15 days, visual observations on rooting and sprouting rates were made without opening the regrowth vessels. At the end of one month, the same final evaluations as for Experiment 1 were conducted. In addition to the above described parameters, mean number of roots per rooted shoot and fresh weights of the plantlets were also measured. The whole experiment was conducted 3 times.

In both experiments, SAS (Statistical Analysis System, S.A.S. Inst. Inc., Cary, NC, USA) GLM (General

Linear Models) procedures were used for data analysis with a Duncan's test (α<0.05).

Results

Experiment 1

After 1 month in the regrowth conditions, 100% of both encapsulated and naked nodes were viable. The naked nodes showed 90% sprouting (i.e., production of shoots longer than 5 mm) and 91.7% rooting. All sprouted nodes had also rooted, therefore the final plantlet regeneration rate was 90%. The shoots had expanded green leaves, with a mean of 1-cm large and 2.5-cm long laminae that were more regularly shaped than those produced during the proliferation stage. All shoots produced callus at their base.

The encapsulated nodes, instead, achieved only 21.3% sprouting. Rooting was approximately 10%. Only 10% of the initially-sown encapsulated nodes produced a plantlet. The sprouting process caused the breakage of the capsule, whose fragments remained visible at the base of the shoots. Very little callus was produced by these sprouted nodes. None of the single nodes that did not show regrowth, broke their capsules. In some cases, the nodes were elongated inside the bead, but had remained entrapped inside the capsule.

Experiment 2

Visual observations on the single nodes after 3 days of root primordia initiation culture did not show any root or shoot formation. After 6 days, 5-10% of the single nodes had small root tips at their base. After 9 days, over 50% of the nodes had small visible root tips, modest elongation of the shoot and a little callus production at the base. In some cases, the singles nodes that had been cultured for root primordia initiation for 9 days were difficult to encapsulate, because of the presence of the callus and of the elongation of the explant, that caused big alginate drops and therefore big capsules.

Visual observations after 15 days from sowing showed that all encapsulated explants were viable. The rooting rate of the single nodes encapsulated after 9 days of root primordia initiation was 86%, significantly higher than that of the single nodes encapsulated after 3 days and 6 days of root primordia initiation culture (30.4% and 42.6%, respectively). Sprouting rates, instead, ranged from 52% to 58%, with no significant differences between the three treatments. Only 26% and 24% of the nodes cultured for root initiation for 3 days and 6 days, respectively, produced callus at the lower cut. In contrast, 40% of the nodes cultured for root initiation for 9 days had produced callus that was much larger than on nodes cultured for 3 days or 6 days.

After one month in the regrowth conditions, all explants of all treatments were viable. The regrowth values ranged from 90% to 94%, with no significant differences between nodes encapsulated after 3 days, 6 days or 9 days of root primordia initiation culture. In all treatments, sprouting percentages showed a significant increase from the 15^{th} to the 30^{th} day in the regrowth conditions. At the end of 1 month, 74% to 81.8% encapsulated nodes had sprouted. The differences between these rates were not statistically significant.

Single nodes cultured for 9 days for root primordia initiation reached the highest rooting percentage (88.0%). No significant differences were monitored between rooting rates of encapsulated nodes cultured for root primordia initiation for 3 days and 6 days (63.3% and 71.6%, respectively). Rooting rate increase from the 15^{th} to the 30^{th} day in the regrowth conditions was significant for nodes encapsulated after 3 days and 6 days of root primordia initiation culture, while it was not significant for the others.

No significant differences between treatments were monitored for mean root number (2-3 roots/node) and plantlet fresh weight (it ranged from 115 to 139 mg/plantlet).

As a consequence of the fact that not all rooted nodes had sprouted, nor that all sprouted nodes also rooted, final plantlet production rates were 58%, 60% and 66% for nodes cultured for root primordia initiation for 3 days, 6 days and 9 days, respectively. The differences between these rates were not statistically significant.

The percentages of single nodes that had produced callus had not changed in the last 15 days in the regrowth conditions. The callus that had been already produced in some of the explants in the first 15 days continued to grow for the last 15 days only in those explants that did not root.

As in Experiment 1, the plantlets formed had expanded leaves, bigger than those of the original shoots from the proliferation medium, and well formed roots (Figure 1). An apparently different behavior was noted between apical tips and axillary buds, with the

Figure 1. Experiment 2. Plantlet from an apical single node encapsulated after 6 days of root primordia initiation culture at the 30^{th} day in the regrowth conditions. (Measure unit = cm)

former rooting and sprouting more quickly and abundantly.

Discussion and conclusions

The auxin treatment used in these experiments proved effective in promoting rooting of M.26 single nodes, since very high rooting rates were obtained. Only when the explants were encapsulated immediately after the auxin treatment, as in Experiment 1, was the rooting rates were low. Welander and Pawlicki (1993) reported that the root primordia initiation process starts after the first 3 days following induction, with the beginning of cell divisions, while the first evidence of root meristemoids occurs after 4-5 days. Preliminary anatomical investigations of auxin-treated M.26 single nodes seem to confirm this (data not shown). Furthermore, Bhattacharya (1988) explained how respiratory enzymes and oxidases are involved in the initial stages of adventitious rooting. The same author pointed out the high oxygen requirements during this phase. Immediate encapsulation after the auxin treatment, such as in Experiment 1, might have caused a gas exchange limitation between the alginate-enclosed single nodes and the environment, and therefore an oxygen deficiency to the tissues, as explained by Hulst et al. (1989). Inhibitory effects on single node regrowth caused by the alginate capsule were also reported in a previous work with M.26 and other species (Piccioni and Standardi, 1995).

The root primordia initiation culture of a few days following the auxin treatment seemed necessary for improving rooting performances of the encapsulated explants. The highest rooting rates were obtained when encapsulation was delayed the most. When encapsulation was made after 9 days from the auxin treatment, rooting performances were similar to those of the nodes kept naked throughout Experiment 1.

The importance of darkness during the root primordia initiation culture is to be evaluated with appropriate experiments. In fact, in Experiment 1 naked nodes did not undergo any darkness period after the auxin treatment, but they gave high rooting rates.

Furthermore, it is important to emphasize that the root primordia initiation culture alone was not enough to promote regrowth and rooting. Preliminary experiments showed that if encapsulation was made with an artificial endosperm containing nutrients alone, such as that used in Experiment 1, plantlet production rates were lower than 8%. In the same preliminary experiments, addition of growth regulators to the artificial endosperm alone, i.e. without root primordia initiation culture, also resulted in unsatisfactory plantlet production rates (data not shown). Those results were in contrast to previous ones obtained with M.26 single nodes, which gave very high sprouting rates after encapsulation with an artificial endosperm identical to that of Experiment 1, with or without the same growth regulators used in Experiment 2 (Piccioni and Standardi, 1995; Piccioni et al., 1996). In those previous studies the explants had not been submitted to any

induction treatment before encapsulation. It must be presumed that the buds were stressed by the auxin treatment immediately followed by encapsulation, so that both rooting and sprouting were partially compromised. Delaying encapsulation and adding growth regulators to the encapsulation matrix, as in Experiment 2, seemed to solve the problem, enhancing rooting and sprouting rates.

Callus production following the root induction treatment is undesirable (Zimmerman, 1984). Since callus was visibly smaller in the encapsulated explants of Experiment 2 than in the naked single nodes of Experiment 1, it seems that the alginate bead reduced callus growth rate. This effect is generally reported for immobilization of plant cells (Barbotin et al., 1993). Furthermore, the negative interaction that was seen between rooting and callus growth is probably related to competition between the two pathways, in which the progression of the rooting process limits cell growth towards unorganized callus. It is known that different morphogenic processes are mutually exclusive (Ammirato, 1985).

In conclusion, even though plantlet production rates were not significantly different within the treatments tested, it must be said that 9 days of root primordia initiation culture caused callus production on many single nodes and excessive growth of the explants before encapsulation. Therefore, shorter root primordia initiation culture periods (3 and 6 days) are suggested. New experiments are in progress to increase plantlet production rates and to evaluate the possibility of sowing the encapsulated apple nodes directly in soil-like substrata, in view of their use for *ex vitro* purposes.

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