Improving Micropropagation of Hazelnut Italian Cultivars through Temporary Immersion System

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Abstract

Temporary immersion techniques for plant in vitro culture, avoiding the time-consuming sub-culturing necessary with the use of solid media have been applied to various species in order to increase the multiplication rate and to automate as much as possible the plant production. In this work in vitro grown shoots of Italian cultivars 'Montebello' and 'Tonda Gentile Romana' of *Corylus avellana* L., were cultured on a solid medium or temporary immersion system using a basal culture medium, previously defined. Different carbon sources (sucrose or glucose) and durations of immersion (30, 60 or 120 minutes of immersion per day) were applied to determine the best cultural conditions for shoot proliferation. The temporary immersion system enhanced shoot multiplication and gave no hyperhidric shoots. Shoots obtained with this system were induced to root by immersion for one day in 80 mg.L^{-1} IBA solution and root expression was performed either in gel rite, agar and/or vermiculite. The most effective treatment in terms of rooting (100% and 80% in 'Montebello' and 'Tonda Romana', respectively) and acclimatisation (up to 100% in 'Montebello') was shown to be the combination of vermiculite with agarised medium. Hazelnut showed a good aptitude to be in vitro multiplied with the temporary immersion system and further studies are now in progress to optimise the protocol in view of a possible wider application of temporary immersion systems (TIS) to this species.

INTRODUCTION

Plant propagation through tissue culture has limitations due to the requirement of highly skilled labour. For this reason, temporary immersion systems (TIS), which can avoid the time-consuming sub-culturing necessary with the use of solid media, was recently assessed in various species including fruit woody species such as apple, pear and Mediterranean small fruit species (Zhu et al., 2005; Damiano et al., 2002, 2007) in order to increase multiplication rates, to automate plant production and to reduce costs for the propagation process.

Micropropagation based on a temporary immersion approach has also been shown to reduce problems usually encountered in permanent liquid cultures such as hyperhydricity and poor quality of propagules (Escalona et al., 2007).

TIS was also proven to be applicable for the efficient and reproducible production of plant pharmaceuticals, being a system particularly suitable for manipulating plant metabolism to generate active compounds (Gerth et al., 2007).

Among the various technical solutions applied for TIS, RITA® System (Teisson and Alvard, 1995), consisting of autoclavable vessels in which the liquid medium is cyclically pushed from an air pump, is one of the most successfully applied (Zhu et al., 2002; McAlister et al., 2002; Ruffoni and Savona, 2005). Other technical devices were also used successfully in other laboratories (Damiano et al., 2002).

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In this work, in vitro grown shoots of Italian hazelnut cultivars were cultured in a temporary immersion system to determine the feasibility of applying this culture system to this species. An improved protocol for rooting of the cultivars is also described in the present paper.

MATERIAL AND METHODS

Plant Material and Culture Conditions

In vitro-propagated cultures, in vitro grown shoots of the Italian cultivars 'Montebello' and 'Tonda Gentile Romana' of *Corylus avellana* L., originated by axillary buds of mature plants, were cultured on a modified DKW (Driver and Kuniyuki, 1984) medium (MOLT) according to Damiano et al. (2005). Benzyladenine (BA), giberellic acid (GA_3) and indole-3-butyric acid (IBA) were also supplemented to the medium at concentrations of 2.0, 0.03 and 0.01 $mg.L^{-1}$, respectively. Sucrose (30 $g.L^{-1}$) and 6 $g.L^{-1}$ agar (B & V, Italy) were also added in this culture phase. The pH of the medium was adjusted to 5.7 before sterilisation by autoclave and cultures were maintained at 24°C under a 16h photoperiod and a light intensity of 40 $\mu mol\ m^{-2}.s^{1}$ provided by cool white fluorescent tubes (Philips TLD - France) and sub-cultured every 3 weeks.

Temporary Immersion

The experiments were conducted in RITA (VITROPIC - France) containers (Fig. 1A) or in a bottle (Fig. 2B) system (Damiano et al., 2002) containing 150 ml of the above MOLT medium. Two carbon sources (sucrose or glucose 30 $g.L^{-1}$) and three durations of medium immersion were compared (30, 60 or 120 minutes of immersion per day). Shoots were also multiplied on a solid medium as a control. Three replications (containers), with 10 single shoot explants each, were prepared for each TI treatment and five vessels (Magenta-Sigma) with six shoots were used for the solid medium. The experiment was repeated twice. Multiplication rates were calculated and shoot quality (presence of hyperhydricity) was observed after 30 days of culture.

Rooting and Acclimatisation

For the rooting experiments, shoots from TIS (best treatment) and from solid culture were previously elongated for 15 days in an agarised MOLT medium supplemented with 0.5 $mg.L^{-1}$ BA and induced to root by immersion of the basal part in 20 $g.L^{-1}$ sucrose solution containing 80 $mg.L^{-1}$ of IBA for 1 day, in darkness, and transferring to a growth regulator-free medium with or without agar (B & V, Italy) or gelrite and with or without vermiculite (1 g:1 ml of medium).

Rooted shoots were transplanted into a mixture of 60% peat and 40% perlite, under plastic tunnel conditions, with 80% humidity, for two weeks. Then, humidity was gradually reduced, after two weeks, to 60% and plants were transferred to the greenhouse.

Eight vessels containing six shoots were used per treatment for the rooting experiments. The rooting percentages were calculated, recording the number of rooted shoots 30 days after transferring to the root-inducing medium. The number of roots per rooted explant was also recorded. Survival (%) of micro-cuttings after acclimatisation was evaluated 60 days after transferring to the greenhouse. Each experiment was repeated twice and used a completely randomised design. Data was subjected to an analysis of variance by Fisher's protected LSD test at P=0.05. Percentage data was transformed to arcsine before analysis.

RESULTS AND DISCUSSION

The temporary immersion system enhanced multiplication and the shoots did not show an increase in hyperhydricity compared to the shoots cultured on agarised medium. The adoption of the shortest immersion period was critical to obtain high proliferation rates in 'Montebello' (Table 1), while the longest immersion period seems to be more suitable

for ‘Tonda Romana’ (Fig. 1). No significant differences were found between sucrose and glucose and between the two technical devices in ‘Montebello’ (Table 1), while in ‘Tonda Romana’ sucrose induced higher multiplication rates than glucose, particularly with the RITA system.

From these preliminary results, hazelnut showed a good potential to be multiplied in vitro with the temporary immersion system. Scaling up for the automation of the micropropagation process seems to be applicable to this species.

The use of vermiculite, in the rooting expression phase, improved rooting percentage and acclimatisation survival (Table 2). The most effective treatment in terms of rooting percentage and number of roots per rooted explant was shown to be the combination of vermiculite with agarised medium, with 100% and 80% of rooting in ‘Montebello’ and ‘Tonda Romana’, respectively (Table 2; Fig. 2). Gelrite as a gelling agent was less suitable for rooting than agar in this species. Addition to the medium of vermiculite strongly enhanced rooting formation and, as previously reported for walnut, apple and papaya (Jay Allemand et al., 1992, Kolozsvari Nagi and Sule, 2006; Yu et al., 2000) the positive effect of the addition of vermiculite to the substrate seems to be related to the increased aeration of the substrate, which induces the formation of roots and makes the plants more able to survive as transplants (Yu et al., 2000). In addition, the acclimatisation of plants induced to root on vermiculite was higher (90%) than those transferred for the expression of the rooting phase, to the agarised or gelrite added medium (Figs. 3, 4; Table 2).

CONCLUSIONS

From these preliminary results, hazelnut showed a good potential to be in vitro multiplied through TIS and further studies are now in progress to optimise the protocol and to characterise the quality of the explants.

In this work we also presented an improved protocol for in vitro rooting of Italian hazelnut cultivars, showing the positive effect of the use of the combination agar plus vermiculite on the rooting response and on the following phase of acclimatisation.

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Tables

Table 1. Multiplication rates obtained in TIS and in solid medium in 'Montebello'.

Propagation methods	Sugar	Immersion period (min.)	Multiplication rates
Solid medium (control)	Sucrose		3.9±0.5
	Glucose		4.2±0.6
RITA device	Sucrose	30	3.9±0.7
	Sucrose	60	2.8±0.4
	Sucrose	120	2.3±0.5
	Glucose	30	5.2±0.4
	Glucose	60	2.9±0.4
	Glucose	120	0.0
Bottle device	Sucrose	30	4.9±0.7
	Sucrose	60	2.1±0.4
	Sucrose	120	1.2±0.4
	Glucose	30	5.4±0.7
	Glucose	60	2.3±0.3
	Glucose	120	0.0

Table 2. Effect of the addition of vermiculite to liquid (LIQ), agarised (AG) o Gelrite (GR) medium on rooting %, number of roots per rooted microcutting (N) and acclimatisation % during rooting expression.

	Rooting %	N	Acclim. %
Vermic + LIQ	41c	1.5c	78b
Vermic + GR	59b	3.0b	88b
Vermic + AG	100a	5.8a	96a
GR	39c	3.6b	67bc
AG	76b	2.2bc	55d

Figures

Fig. 1. Cv. 'Tonda Romana'. Shoots in RITA device with 120 min of immersion time/day.

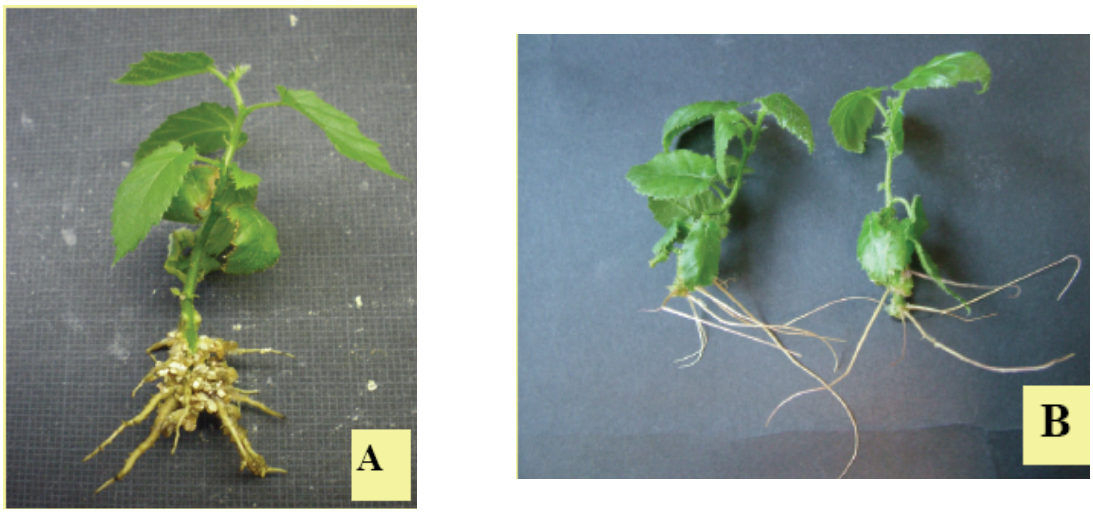

Fig. 2. 'Montebello'. Microcuttings rooted with Agar + Verm. (A) and only with agar (B).

Fig. 3. Acclimatisation of ‘Montebello’ and ‘Tonda Romana’ under plastic tunnel.

Fig. 4. Acclimatised plants. A. ‘Tonda Romana’ rooted with agar + vermiculite. B. ‘Montebello’ rooted with agar + vermiculite. C. ‘Montebello’ rooted with only agar.