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Micropropagation of *Ilex glabra* (L.) A. Gray

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***Abstract*. Nodal segments containing one axillary bud (1 to 1.5 cm) were disinfected using 10% bleach and were established on a Murashige and Skoog (MS) medium without hormones at 27 °C and with a 16-h photoperiod. The sprouted shoots (≈1.0 cm) were cultured on a MS medium supplemented with 6-benzylaminopurine (BAP), kinetin (KIN), or zeatin (ZT) at 2.3, 4.5, 9.1, or 18.2 μM. After 38 d, ZT and BAP significantly induced multiple shoot formation with multiplication rates of 4 to 6, whereas the multiplication rate of KIN was less than 2. Shoots cultured on ZT grew significantly taller than those on BAP and KIN. The height of the longest shoots treated with ZT was 4.6 cm, which was 1.6 to 2.2 times greater than those treated with BAP or KIN. To induce rooting, shoots (≈2 cm) were subcultured on one-fourth strength MS (1/4 MS) medium containing either 3-indolebutyric acid (IBA) or 1-naphthylacetic acid (NAA) at 2.6, 5.1, or 10.3 μM. Adventitious roots formed in vitro after 2 to 4 weeks. IBA at 10.3 μM produced the best rooting (100%) compared with other treatments after 38 d of culture. The average number of roots per shoot for IBA was ≈15, which was 1.6 to 3.1 times as many as that of other treatments. All rooted plantlets were then transplanted into a mix of peatmoss and perlite (1:1 v/v) and acclimatized in a mist system. Average plantlet survival was 73.6% after 35 d. After acclimatization, they were grown in a pot with Metro-mix under greenhouse conditions for 10 weeks where 95% of plants survived and grew up to 6.8 cm high. The micropropagation procedure, i.e., nodal segments containing one axillary bud proliferated on MS with 4.5 μM ZT followed by in vitro rooting on 1/4 MS plus 10.3 μM IBA, could be used for commercial mass production of new inkberry cultivars.**

Ilex, a member of the Aquifoliaceae (holly family), comprises more than 500 deciduous and evergreen shrubs or trees with economic importance as crops and ornamentals in tropical and temperate regions of the world (Galle, 1997; Hu, 1989). *Ilex glabra* (L.) A. Gray (inkberry) is a native evergreen shrub with glossy green foliage and black berry-like drupes. This species grows to a mature height of 1.8 to 2.4 m and a width of 2.4 to 3.0 m (Dirr, 1998). It is a desirable ornamental plant for colder regions. Conventional seed germination and vegetative propagation are two major procedures for mass propagation. However, similar to other *Ilex* species, seed germination of inkberry is inefficient as a result of low germination rate, long germination time, and seedling variation. It may take 2 to 3 years to overcome the double dormancy caused by hard, impermeable seedcoat and immature embryos (Dirr and Heuser, 1987). In addition, vegetative propagation of inkberry by cuttings may be subject to seasonal variations.

Tissue culture is a well-known and efficient tool for mass propagation of uniform plants. In vitro culture of zygotic embryos for propagation of various *Ilex* species plants from rudimentary embryos has been reported (Hu, 1975, 1976; Hu et al., 1979; Mattis et al., 1995; Sansberro et al., 1998, 2001a). They focused on the influences of different factors on overcoming dormancy in rudimentary embryos. Hu (1989) reported that cell suspension cultures and somatic embryos were obtained from immature *Ilex aquifolium* L. (English holly) zygotic embryos; however, the conversion rate of embryos into plants was low. In general, *Ilex* species have been propagated by shoot proliferation using nodal segments, shoot tips, and/or apical meristems (Bernasconi et al., 1998; Luna et al., 2003; Majada et al., 2000; Mattis et al., 1995; Morte et al., 1991; Sansberro et al., 1999, 2000, 2001b; Zaniolo and Zanette, 2001).

Because there is no reported protocol for mass propagation of inkberry using tissue culture, our evaluation of this procedure includes the study of the effects of plant growth regulators on shoot proliferation and rooting.

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Materials and Methods

Plant material and culture establishment. A 1-year-old plant of *Ilex glabra* 'Pretty Boy' (Rarefind Nursery, Jackson, NJ) maintained under greenhouse conditions was used as the source of explants. On 2 June 2008, young, nonlignified branches were collected, surface-disinfected in 70% ethanol for 10 s, then immediately transferred into 10% Ultra bleach (6.0% sodium hypochlorite; Wal-Mart Stores, Inc., Bentonville, AR) with seven drops of Tween 20 (ACC00528/0030; Agdia® Inc., Elkhart, IN) per 200 mL for 10 min, and washed with three to five rinses of sterile distilled water. The branches were dissected into 1- to 1.5-cm-long segments of stem containing a single bud (Fig. 1A). The nodal segments were transferred to 60-mL Pyrex glass tubes containing Murashige and Skoog (MS) (1962) basic medium plus 90 mM sucrose and 8 $g \cdot L^{-1}$ agar for shoot induction. The pH of the media was adjusted to 5.8 ± 0.5 with NaOH or HCl before adding agar (Sigma Chemical Co., St. Louis, MO). A total of 10 mL of the media was transferred by pipette into glass tubes, which were covered with caps and autoclaved at 121 °C for 30 min. All tubes with explants were loaded into tube racks and placed in a plastic bag. They were cultured in a growth room at a temperature of 27.2 ± 1.9 °C with a 16-h photoperiod (138 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density from cool-white fluorescent lamps). After 2 months, axillary shoots had elongated up to 4 cm (Fig. 1B). Nodal segments (≈1.0 cm long) were excised from these axillary shoots and used for the following experiments.

Effects of cytokinins on multiple shoot proliferation. To determine the optimal conditions for both axillary bud proliferation and shoot elongation, 6-benzylaminopurine (BAP), kinetin, 6-furfurylaminopurine (KIN), or zeatin, 4-hydroxy-3-methyl-trans-2-butenylaminopurine (ZT) (all three obtained from Sigma Chemical Co.) at 2.3, 4.5, 9.1, or 18.2 μM were compared. Nodal segments (≈1.0 cm long) were excised and cultured on an MS medium plus 90 mM sucrose and one source and rate of cytokinin. Nodal segments were also cultured on the same medium lacking any cytokinin. All cultures were incubated under the same physical conditions as described previously. A total of 36 glass tubes (replications) with one explant per tube were used for each treatment. After 38 d of culture, total number of shoots (greater than 1.0 cm long) and the length of the tallest shoot were recorded.

Root induction. Terminal shoots (≈2.0 cm long) were rooted in one-fourth strength MS (1/4 MS) medium plus 90 mM sucrose with either 3-indolebutyric acid (IBA) or 1-naphthylacetic acid (NAA) (both obtained from Sigma Chemical Co.) at 2.6, 5.1, or 10.3 μM. Shoots were also rooted on MS and 1/4 MS medium without auxin. A total of 36 glass tubes with one explant per tube were used for each treatment. All cultures were incubated in the same physical conditions as described previously. After 38 d of culture, the number of explants with

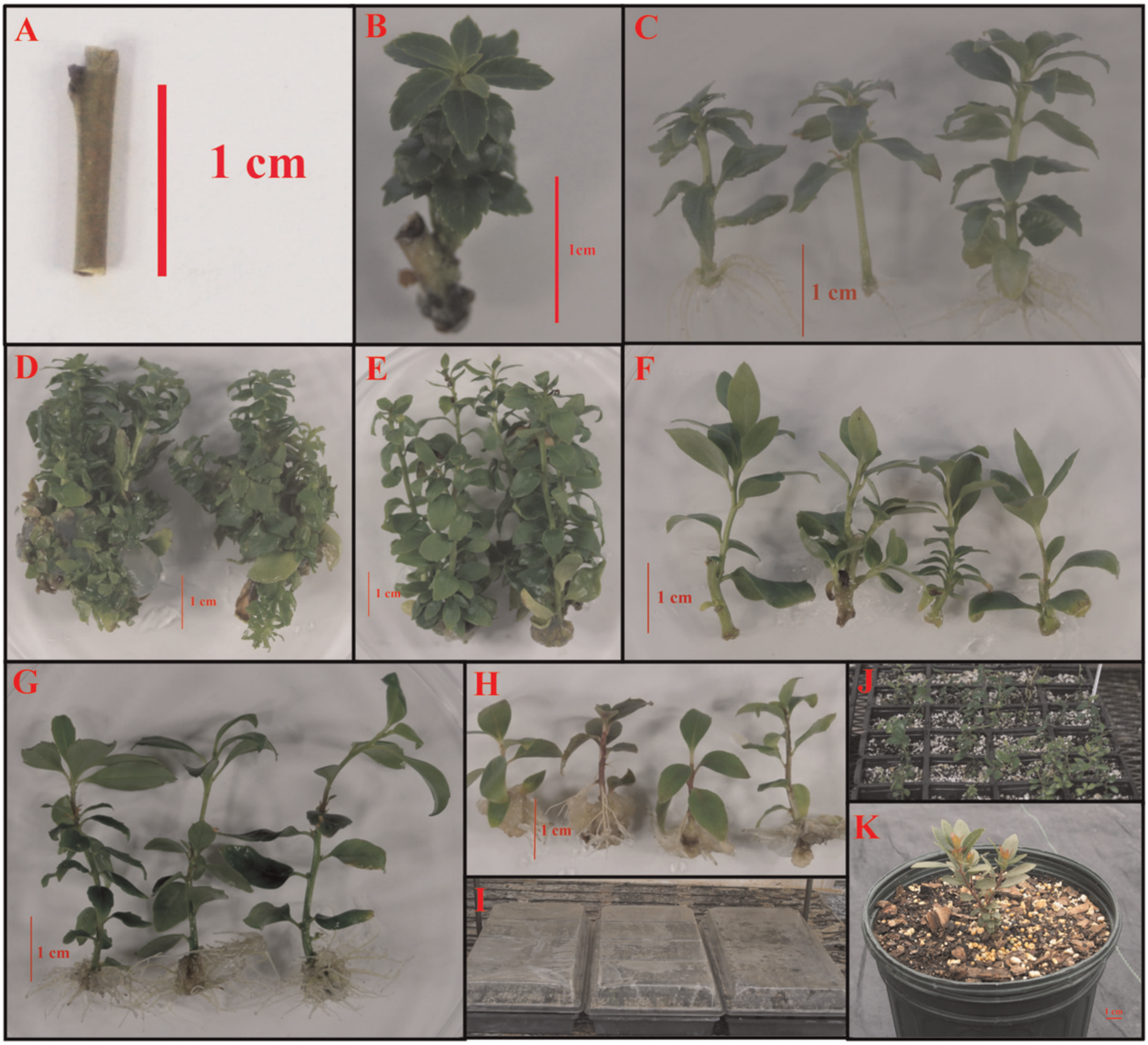

Fig. 1. Morphogenetic responses of *Ilex glabra* during the establishment (**A–B**), multiplication (**C–F**), rooting (**G–H**), acclimatization (**I–J**), and transplanting (**K**) stages. (**A**) Nodal segment containing one axillary bud collected as explants; (**B**) explants established on Murashige and Skoog (MS) medium plus 90 mm sucrose; (**C**) shoots cultured on MS medium without cytokinin; shoots proliferated and elongated on MS medium supplemented with 4.5 µM 6-benzylaminopurine (BAP) (**D**), 4.5 µM zeatin (ZT) (**E**), or 4.5 µM kinetic (KIN) (**F**); (**G**) microcuttings rooted in vitro on 1/4 MS medium plus 10.3 µM 3-indolebutyric acid (IBA); (**H**) roots and callus tissue formed on 1/4 MS medium plus 10.3 µM 1-naphthylacetic acid (NAA); plantlets rooted in vitro acclimated using dome for first 2 weeks (**I**), and then kept under mist for 2 more weeks (**J**), (K) Acclimated plantlet transplanted into pots.

roots, the number of roots per explant, and the length of the longest root on each explant were recorded.

At the same time, in a separate trial, terminal shoots (3 to 4 cm long) obtained during the multiplication stage were also dipped in a 5.1 µM IBA solution for 20 s, inserted in a flat with $5.0 \times 5.0 \times 6.0\text{-cm}^3$ cell, which contained perlite (Whittemore Company Inc., Lawrence, MA), and a commercial substrate (PRO-MIX BX, which contains 65% to 75%, by volume, of Canadian sphagnum peatmoss; 35% to 25% of perlite, vermiculite, macronutrients and micronutrients, dolomitic, and calcitic limestone; Scotts Sierra Horticulture Products Co., Marysville, OH) in a ratio of 1:1 by volume. Flats were placed under intermittent mist. Misting frequency was controlled by a timer (Phytotronics Inc., Earthcity, MO) set at 20 s every 10 min for the first 2 weeks and then reduced to 20 s every 20 min for the remainder of the experiment. Mist system was on in the morning and off in the evening. No additional light was provided. The number of surviving plantlets, the number of explants with roots, the number of roots per explant, and the length of the longest root were recorded.

Acclimatization and transplanting. On 10 Oct. 2008, the plantlets obtained in vitro were removed from the culture tubes and their roots carefully washed under tap water to remove the agar medium. They were transferred to a tray with 32 cells ($5.0 \times 5.0 \times 6.0$ cm^3) filled with a mixture of PRO-MIX BX as described previously and perlite (1:1 v/v). During transplanting, relative humidity (RH) was maintained 100% using a Ultrasonic Whisper Quiet Cool Mist Humidifier (SU-2000; Sunpentown International Inc., Industry, CA) to avoid dehydration. Plantlets in a tray were covered with a clear, plastic dome and acclimated (25.4 ± 1.7 °C, 100% RH) under a mist system in a greenhouse for the first 2 weeks. The domes were removed and plantlets were then kept under the mist system described previously for 2 more weeks. By lengthening the interval between misting, the RH was gradually decreased from 100% to ≈70%. After 4 weeks of acclimatization, the percentage of surviving plants was recorded. They were transplanted into 3.3-L plastic pots with a commercial substrate containing 35% to 45% bark, 20% to 30% coir, 10% to 20% Canadian sphagnum peatmoss, 5% to 15% horticultural-grade perlite, 5% to 15% processed bark ash, starter nutrient charge, dolomitic limestone, and our long-lasting wetting agent (Metro-Mix 560 Coir; Scotts-Sierra Horticultural Products Company) and grown in a glass greenhouse (22.6 ± 6.2 °C). Plant survival after transplant and the plant height (shoot length) were also recorded 7 weeks later.

Experimental design and statistical analysis. A completely random design was used in all cases. A total of 36 explants were cultured per treatment. The treatments were arranged randomly on the shelves in the growth room. The results presented for multiple shoot proliferation were the means of 36 explants with ± SE, whereas those for rooting experiments were the means of three individual experiments with ± SE 36 explants per experiment. Because ex vitro rooting experiments were performed with 3- to 4-cm shoots, whereas in the other experiments, 2-cm shoots were used, data of ex vitro rooting were omitted from the analysis. Data on full-strength MS medium were also excluded from the analysis because auxin was not added. The square root of original data was used for analysis. A two-way analysis of variance (ANOVA) was performed using Statistical Analysis Systems (SAS Version 9.1; SAS Institute, Inc., Cary, NC). Student-Newman-Keuls test at $P < 0.05$ was applied for means separation. Linear trend analysis for each cytokinin was also conducted.

Results and Discussion

The effects of cytokinin source and rate on in vitro shoot proliferation of inkberry single-node explants are summarized in Table 1. Shoot proliferation occurred to varying degrees on all tested media. Both the type and concentration of the cytokinins significantly affected the number of shoots per explant and their height. ANOVA also demonstrated a significant interaction between both factors. Explants on MS medium without cytokinin (control) produced few shoots and developed many adventitious roots (Fig. 1C). BAP at all four concentrations and ZT at 4.5, 9.1, or 18.2 μM significantly increased the number of shoots per explant compared with the control (no cytokinin) (Fig. 1D–E). KIN at all tested concentrations and ZT at 2.3 μM did not stimulate shoot proliferations (Fig. 1F). The range of shoot number per explants was 3.8 to 5.5 on media containing 2.3, 4.5, 9.1, or 18.2 μM BAP or on media containing 4.5, 9.1, or 18.2 μM ZT but less than 2.3 on that with KIN or 2.3 μM ZT (Table 1). Our results are in general agreement with previous reports for English holly (Majada et al., 2000) and *Ilex dumosa* var. *dumosa* Reissek (Luna et al., 2003), although they reported BAP was a better cytokinin for shoot proliferation than ZT. The response to the increasing KT and ZT concentrations was linear ($P < 0.0001$ for both KT and ZT), but not for BAP ($P = 0.11$). After 38 d of culture, the highest number of shoots per explant (5.5) was produced when nodal segments were grown on MS medium supplemented with 9.1 μM BAP or 18.2 μM ZT. This number was higher than that reported with English holly (Majada et al., 2000), *Ilex dumosa* var. *dumosa* (Luna et al., 2003), and *Ilex paraguariensis* A. St. Hil. (Sansberro et al., 1999; Zaniolo and Zanette, 2001) in which an average of four shoots per explant was obtained with 4.5 μM BAP. ZT, at all four tested concentrations, and BAP, at 2.3 μM, increased shoot growth, which ranged from 4.2 to 4.6 cm, compared with the control (2.6 cm). However, the heights of the tallest shoot on 4.5, 9.1, or 18.2 μM BAP and all tested KINs were less than 2.8 cm (Table 1) and not significantly different from that of the control. Although different basic medium was used, these results were still supported by those reported for English holly (Majada et al., 2000). They reported that the length of axillary shoots on woody plant (Lloyd and McCown, 1980) medium containing with 2.3 and 9.1 μM ZT was higher than that with BAP and KT. The response to the increasing BAP ($P < 0.0001$) and KIN ($P = 0.013$) concentrations was linear, but that was not the case for ZT ($P = 0.97$). On the basis of these results and the cost of the cytokinins, a concentration of 4.5 μM ZT is most appropriate for shoot proliferation of inkberry.

Adventitious roots initiated after shoots were cultured ≈2 weeks on all media supplemented with IBA or no auxin (Fig. 1G); the percentage of rooted explants ranged from 10% to 39% (data not shown). Similarly, Majada et al. (2000) reported that roots of English holly formed after 2 weeks of culture. However, rooting of *Ilex dumosa* var. *dumosa* took ≈4 weeks (Luna et al., 2003). Shoots on all media containing NAA began to root after 3 weeks in culture (data not shown) and these shoots had high basal callus formation (Fig. 1H). After 38 d of culture, much higher rooting percentage was observed on all 1/4 MS media compared with full-strength MS media and ex vitro rooting (Table 2). Compared with 1/4 MS

Table 1. Effect of cytokinin source and rate on in vitro multiple shoot production of *Ilex glabra* single-node explants after 38 d of culture on Murashige and Skoog media supplemented with 90 mM sucrose.

Hormone	Concn (μM)	No. of shoots/explant[z]	Ht (cm)[z]
Control	0	1.2 ± 0.1c[y]	2.6 ± 0.1 bc
BAP	2.3	4.2 ± 0.4 b	4.4 ± 0.2 a
	4.5	3.8 ± 0.3 b	2.6 ± 0.2 bc
	9.1	5.5 ± 0.6 a	2.8 ± 0.2 b
	18.2	4.8 ± 0.7 ab	2.1 ± 0.1 c
KIN	2.3	1.5 ± 0.1 c	2.1 ± 0.1 c
	4.5	1.7 ± 0.1 c	2.1 ± 0.2 c
	9.1	1.7 ± 0.2 c	2.5 ± 0.2 bc
	18.2	2.3 ± 0.2 c	2.5 ± 0.1 bc
ZT	2.3	2.3 ± 0.2 c	4.2 ± 0.2 a
	4.5	3.9 ± 0.3 b	4.6 ± 0.2 a
	9.1	4.4 ± 0.3 ab	4.2 ± 0.2 a
	18.2	5.5 ± 0.4 a	4.4 ± 0.2 a

[z]Values are means ± SE of 36 explants.
[y]Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation and analysis of variance.
BAP = 6-benzylaminopurine; KIN = kinetin, 6-furfurylaminopurine; ZT = zeatin, 4-hydroxy-3-methyl-trans-2-butenylaminopurine.

Table 2. Effect of auxin source and concentration on in vitro rooting of *Ilex glabra* microcuttings after 38 d of culture on one-fourth strength Murashige and Skoog (MS) media plus 90 mM sucrose.[z]

Media	Hormone[z]	Concn (μM)	% Rooting[y]	No. of Roots	Length of roots (cm)
1/4 MS	—	0	63.9 ± 1.1 b[x]	5.4 ± 0.4 c	1.4 ± 0.1 b
1/4 MS	IBA	2.6	58.3 ± 2.8 b	5.3 ± 0.6 c	0.5 ± 0.1 c
1/4 MS	IBA	5.1	66.7 ± 2.3 b	14.9 ± 1.1 a	1.9 ± 0.2 a
1/4 MS	IBA	10.3	100 ± 0.0 a	14.8 ± 1.2 a	1.3 ± 0.1 b
1/4 MS	NAA	2.6	55.6 ± 2.3 b	9.3 ± 0.6 b	0.6 ± 0.1 c
1/4 MS	NAA	5.1	74.4 ± 0.8 b	7.8 ± 0.6 bc	1.3 ± 0.1 b
1/4 MS	NAA	10.3	75 ± 0.6 b	6.8 ± 0.6 bc	1.1 ± 0.1 b
MS	—	—	33.3 ± 0.9	4.9 ± 0.8	1.4 ± 0.1
	Ex vitro rooting[w]		37.6 ± 1.3	3.7 ± 0.2	5.0 ± 0.3

[z]In vitro rooting of *Ilex glabra* microcuttings on full-strength MS media plus 90 mM sucrose and ex vitro rooting are presented as a reference but were excluded from the analysis.
[y]Values are mean ± SE of three individual experiments, 36 explants per experiments; the percent is the percentage of explants with any visible roots.
[x]Different letters in each column indicate that they are significantly different ($P \leq 0.05$) according to Student-Newman-Keuls mean separation.
[w]Microcuttings were dipped in 5.1 μM IBA solution for 20 s, inserted in a medium containing perlite and PRO-MIX BX (65% to 75% of Canadia sphagnum peatmoss; 35% to 25% of perlite, vermiculite, macronutrients and micronutrients, dolomitic, and calcitic limestone) in a ratio of 1:1 by volume, and then kept under mist.
IBA = 3-indolebutyric acid; NAA = 1-naphthylacetic acid.

media without auxin, there was no significant difference among the media with IBA or NAA with exception of the 1/4 MS plus 10.3 μM IBA (Table 2). The highest percentage of rooted shoots (100%) was achieved using this highest concentration of IBA (Table 2). These results were in agreement with previous reports on the rooting of English holly (Majada et al., 2000) and *Ilex paraguariensis* (Zaniolo and Zanette, 2001), although their rooting percentages were lower. On the other hand, Morte et al. (1991) reported that a greater percentage of English holly explants produced roots when pretreated with 0.5 mM NAA (50%) compared with 0.5 mM IBA (15%). It is possible that IBA produced lower rooting percentages in their study because it was not incorporated into the media.

Both the kind and concentration of auxin significantly affected the number and length of roots per explants. A significant interaction between both factors was also observed. The number of roots per explant on 1/4 MS plus 5.1 or 10.3 μM IBA was significantly higher than other treatments (Table 2; Fig. 1G). Both 5.1 and 10.3 μM IBA treatments induced 1.6 to 3.1 times more roots than other treatments. Compared with in vitro rooting, ex vitro rooting produced fewer roots; however, ex vitro roots grew much longer. In addition, prolific root hairs were noted on the main roots of ex vitro roots. Among all in vitro treatments, 1/4 MS plus 5.1 μM IBA produced much longer roots than other treatments, whereas roots on 1/4 MS plus 2.6 μM IBA or NAA were much shorter. However, no significant difference was observed among 1/4 MS, 1/4 MS plus 10.3 μM IBA, 1/4 MS plus 5.1 μM NAA, or 1/4 MS plus 10.3 μM NAA. This may result from either different root initiation or inhibitory effects of auxin on root growth, or both. As a result of a higher percentage and greater number of roots forming, in vitro rooting using 1/4 MS plus 10.3 μM IBA is recommended.

All in vitro-rooted plantlets were pooled and transplanted into a mixture of peatmoss:-perlite (1:1 v/v) and acclimated in a mist system. After transplantation, some of the in vitro-rooted plants died immediately, whereas others died during acclimatization. After 35 d, 73.6% ± 1.8% of the plantlets survived. This was much higher than ex vitro-rooted plants (34.5% ± 3.0%). The original in vitro-rooted plants continued to elongate after transplanting and newly formed roots had morphology similar to that of plants rooted ex vitro. Root hairs on the newly formed roots decreased the imposed stress during and after acclimatization. After 10 weeks in a greenhouse, 95% ± 2.9% of plants that survived initial acclimation survived and grew up to 6.8 ± 0.3 cm high. This yielded a final efficiency of 70%, which was much higher than ex vitro rooting (13.5%). This was also higher than those in previous reports (Majada et al., 2000; Morte et al., 1991), in which a final efficiency of 64% or 40% to 60% was obtained.

Previous studies achieved rooting induction from shoots in two steps (Luna et al., 2003; Majada et al., 2000; Morte et al., 1991; Sansberro et al., 2001b; Zaniolo and Zanette, 2001). They treated the shoots or microcuttings with liquid hormone by quick dipping or cultured them on a medium with rooting hormone for ≈1 week, then cultured or subcultured them on a medium devoid of hormones. This method is time-consuming and expensive. Majada et al. (2000) also reported that higher efficiency was achieved from ex vitro rooting of English holly (80%) than in vitro rooting (64%), and the survival rate of successfully in vitro or ex vitro-rooted plants was not significantly different. However, in our study, ex vitro rooting was not efficient. The highest efficiency for propagation was obtained with in vitro rooting (70%). Less than 50% of the explants of *Ilex paraguariensis* formed roots when they were cultured on 1/4 MS medium supplemented with 51.5 to 76.5 μM IBA (Sansberro et al., 2001b). Generally, rootless microcuttings may die in larger numbers when removed from culture, whereas rooted microcuttings have a higher survival rate. Because a great number of unrooted microcuttings were lost and low rooting percentages were obtained during ex vitro rooting, in vitro rooting and acclimatization appears to be more appropriate.

In conclusion, a protocol for in vitro propagation of inkberry from juvenile explants is: Stage I, surface-sterilized single nodal segments implanted on MS medium plus 90 mM sucrose (Fig. 1B); Stage II, shoots proliferated and elongated on MS supplemented with 4.5 μM ZT (Fig. 1E); Stage III, in vitro rooting on 1/4 MS plus 10.3 μM IBA (Fig. 1G); and Stage IV, acclimatization in a mist system (Fig. 1I–J) followed by transplanting into growing medium (Fig. 1K).

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