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Scientia Horticulturae 103 (2005) 479–488
SCIENTIA HORTICULTURAE
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Effect of leaf retention and flavonoids on rooting of *Ilex paraguariensis* cuttings

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Received 27 August 2002; received in revised form 15 June 2004; accepted 20 July 2004

Abstract

The effect of the mature leaf retention and the exogenous application of flavonoids (naringenin, quercetin and rutin at 30 μM for 12 h) was studied for adventitious rooting of *Ilex paraguariensis* cuttings. Softwood cuttings harvested from young 3-year-old plants and adult 10- and 20-year-old plants were rooted under intermittent fog. A strong correlation ($r^2 = 0.72$) between leaf retention and rooting was noted. The highest percentage of adventitious root formation (40%) was obtained when the leaf was artificially removed after 42 days of incubation. This data was supported by the histological analysis which provided anatomical evidence that cuttings have initiated root primordia by 21 days and the regenerated roots emerge through the epidermis after 35 days of incubation. A strong correlation between the position of the leaf and the site of roots regeneration was observed. A 100% of the rooted cutting with a single leaf only formed roots along the leaf axis at the base of the cutting. Quercetin increased the rooting percentage more than three times compared to the control and all flavonoids tested improved the distribution of roots around the stem without impacting the number of regenerated roots per rooted cutting from 20-year-old plants.

Keywords: *Ilex paraguariensis*; Yerba mate; Vegetative propagation; Adventitious rooting; Leaf retention; Flavonoids

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doi:10.1016/j.scienta.2004.07.004

1. Introduction

Ilex paraguariensis St. Hil. (“yerba mate”) is a dioecious evergreen tree native of South America (Giberti, 1995). Like tea, the leaves and young shoots are rich in caffeine and teobromine (Filip et al., 1998). Therefore, they are consumed as an infusion or decoction. Because of its allogamy, asexual reproduction would be of great value for the multiplication of select clones. Successful propagation is possible for juvenile material (Sansberro et al., 1999); however, like many other woody species, mature tissues show a low morphogenetic potential, which makes it difficult to clone mature trees by rooting cuttings or by in vitro techniques. This is a major obstacle in yerba mate improvement work, because the characteristics of the mature trees cannot always be predicted from traits displayed during the juvenile phase (Mroginski et al., 1997). Although some factors associated with adventitious rooting such as type, age, and size of the cuttings and substrate composition, hormone, and antioxidant treatments were previously studied (Cortezzi Graca et al., 1988; Caso and Dotta, 1997), the physiological and biochemical processes underlying maturation remain still unknown.

Previous observations had revealed that stem browning and subsequent death of yerba mate cuttings are associated with the lack of leaf retention. The purpose of the present study was to study the effect of leaf retention and exogenous flavonoids on adventitious root formation under in vivo conditions using stem cuttings from young and adult *Ilex paraguariensis* plants.

2. Materials and methods

2.1. Plant material

Shoots (35–40 cm long) were harvested from young 3-year-old and adult 10- and 20-year-old stock plants grown either in a greenhouse or in the field. In both cases, the plants were severely pruned in early spring. The shoots were carried to the lab in polyethylene bags at 12–15 °C and were cut just below a node to create a cutting of approximately 10–12 cm long (0.3–0.5 cm diameter). In all experiments, the leaves were cut in half transversely. For most experiments the cuttings consisted of six to nine nodes in which the uppermost mature leaf was retained and the lower six to eight leaves were removed.

2.2. Experiments related to the effects of leaves

In order to study the effect of leaf retention on rooting process, three different experiments with cuttings from 10-year-old plants were carried out. (1) The relationship between leaf abscission and the cutting death by stem browning was firstly determined using one-leaved cuttings. Both parameters were recorded weekly for the 63-day-experiment. (2) The effect of the period of leaf retention on adventitious rooting was investigated using one-leaved cuttings and the required time for root initiation was determined. In this case the leaf was removed (“artificial abscission”) on days 1, 7, 14, 21, 28, 35 or 42. At the same time, histological observations were made from the control cutting in which one leaf was retained throughout the entire period of 42 days. Rooting percentage was assessed after 6 weeks. In all experiments, the 15 mm-basal portion of the cuttings was immersed in 15 mM ascorbic acid solution for 12 h and dipped in a talc mixture containing 1.5% 3-indolebutyric acid (IBA), 2% 1-phenyl 3-methyl pyrazol (PPZ)—5-ona, 10% sucrose, and 10% Captan fungicide (Caso and Dotta, 1997). (3) Finally, a third experiment was conduced in order to compare the frequency and distribution of the adventitious roots from cuttings with 1, 2, 4 or 6 leaves. In this case, cuttings were prepared with either 1, 2, 4 or 6 leaves present. Rooting percentage and the number of roots per rooted cutting were assessed after 8 weeks.

2.3. Treatments with flavonoids

Shoots were collected from 3-year-old plants and 20-year-old plants grown in a greenhouse and cuttings were prepared with or without a single leaf as described above. This time, the cuttings were dipped in an aqueous solution of 30 μM naringenin, quercetin or rutin (Sigma Chem. Co., USA) in replacement of ascorbic acid for 12 h. In the control treatment, the cuttings were dipped in distilled water. In the case of the defoliated cuttings, the leaf was removed after immersion in the tested aqueous solutions. Rooting percentage and number of roots per rooted cutting were assessed after 8 weeks.

2.4. Experimental conditions

The cuttings were set into trays containing 24 × 150 ml cavities filled with perlite plus 0.5 g of controlled release micro-fertilize (Osmocote®, N, P, K; 18, 5, 9, 180-day-release) per cavity. They were grown for 6–8 weeks in a growth chamber, made of polycarbonate and aluminum, providing a day/night air temperature of 25–27/20–22 °C and substrate temperature of 22–25 °C. Relative humidity was maintained at 90% during the first 7 days by a fog device and then decreased gradually until 70%. A 14 h photoperiod was kept throughout the rooting period using 20% sunlight radiation (150–180 μmol m^{-2} s^{-1}, PPFD in the wavelength range of 400–700 nm) plus 100 μmol m^{-2} s^{-1} PPFD using eight cool-white fluorescent lamps (40 W) set at 1.8 m over the cuttings and outside the growth chamber.

The experiments were done during autumn in the years, 1999–2001.

2.5. Statistical analysis

Each treatment consisted of 12 or 24 cuttings and the experiments were repeated three times. Data were subjected to ANOVA (GraphPad Software, San Diego, CA) following Tukey's multiple comparison test and regression analysis. To assess statistical significance, a probability level of 0.05 was chosen.

2.6. Ontogenetic analysis of root primordia

Thirty-five cuttings were taken and split into seven sets of five replicates. The 0.6–3.5 cm-basal portion of the cuttings were removed and fixed in a formalin:ethanol:acetic acid (FAA) solution and dehydrated with Biopur® series. Transverse and longitudinal serial sections of 18–20 μm thick were stained with safranin—Astra blue and mounted in Canada balsam. The photomicrographs were taken with an OLYMPUS CH30 photomicroscope to which a SONY ExwaveHAD camera had been adapted. Samples were taken on 1, 7, 14, 21, 28 and 35 days after the beginning of the rooting process.

3. Results

3.1. Interactions between leaves and adventitious rooting

The pattern of the leaf abscission (ranged from 0 to 49.3 ± 8.2%) and death of the one-leaf cuttings (ranged from 0 to 40.8 ± 4.4%) from 10-year-old plants were significantly related with the course of time (r^2 = 0.76 and 0.82, respectively) and showed a strong correlation between them (Pearson $r = 0.92$, $P = 0.0001$). Likewise, a strong relationship ($r^2 = 0.72$) between leaf retention and adventitious rooting was also found and the highest rooting percentage was obtained when the leaf was artificially removed after 42 days of incubation (Fig. 1). This data was supported by the histological analysis (Fig. 2A–G) which provided anatomical evidence that cuttings have initiated root primordia by 3 weeks and the regenerated roots emerge, through the epidermis, during the course of the 5th week. In fact, after 1 week of induction, the site of root initiation is formed by successive periclinal and anticlinal division of cambial cells. The early events were characterized by the appearance of a small group of cells with nuclei intensely stained in red due to their high mitotic activity (Fig. 2B), which leads on meristem formation by means of successive divisions in diverse planes and enlargement of the resulting cells. As a consequence of further growth, the meristem developed into root primordia during the 3rd week (Fig. 2C–D). The last step

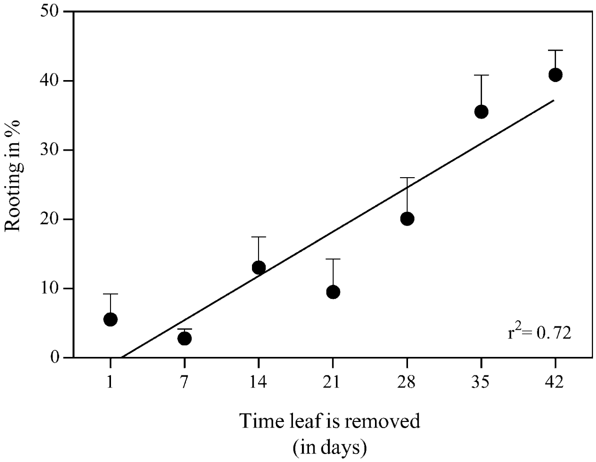

Fig. 1. Effect of leaf retention on adventitious rooting of one-leaf cuttings, harvested from 10-year-old plants. Values are mean ± standard errors. The solid line was obtained by linear regression fitting to the whole data set.

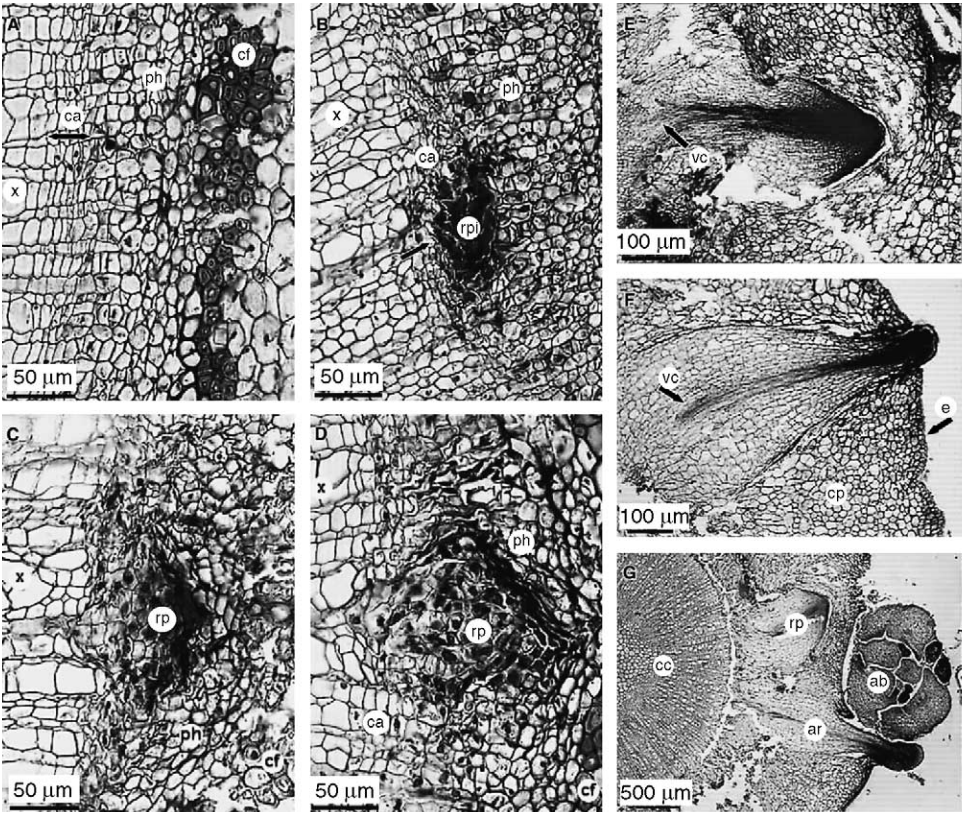

Fig. 2. Initiation and development of adventitious root primordium in *Ilex paraguariensis* cuttings taken from 10-year-old plants. It shows that root primordia developed in the region where the first cell division occurred: (A) transversal sections showing a different stem tissue on day 1; (B) cell divisions of recent derivatives from the cambium give rise to a root primordium initial after 1 week of induction; (C) and (D) early development stages of root primordium during the third week; (E) direct vascular connection are differentiating between the root and stem vascular system; (F) and (G) the adventitious roots emerged after 5 weeks of incubation (ab: axillary bud; ar: adventitious root; e: epidermis; ca: cambium; cc: central cylinder; cf: cortical fibers; cp: cortical parenchyma; ph: phloem; rpi: root primordium initials; rp: root primordium; vc: vascular connections; x: xylem).

is the establishment of a direct vascular connection between the developing root and the stem vascular system (Fig. 2E) and it occurred before the time of root emergence through the stem. The presence of a continuous sclerenchyma ring (Fig. 2A) exterior to the point of origin was not a physical barrier to root emergence.

The number of leaves retained by a cutting was more important than the initial number of leaves per cutting (Table 1). In fact, 79% of cuttings that started with one leaf and retained that leaf rooted at 40%, while 61 and 68% of cuttings that started with two or four leaves retained at least one leaf (same as the one-leaf cutting) rooted at only 17 or 7%, respectively. This was probably as a result of a more stress situation that cause leaf abscission that leads to poor rooting.

Table 1
Leaf retention (as percentage of cuttings with all leaves) scored at the end of the rooting process in evergreen cuttings harvested from 10-year-old plants

Number of mature leaves per cutting at the beginning of rooting process	Number of leaves retained at the end of the rooting process						Rooting (%)	Roots per rooted cutting
	0	1	2	3	4	6		
One leaf	21 ± 2.4	79 ± 2.4	–	–	–	–	40.3 ± 10 a	7.8 ± 0.8
Two leaves	16.6 ± 9.5	61.1 ± 14.8	22.3 ± 11.1	–	–	–	16.7 ± 4.2 ab	12.1 ± 2.3
Four leaves	0	68.1 ± 10.8	16.6 ± 16.6	15.3 ± 9.7	0	–	7 ± 2.8 b	10.4 ± 0.7
Six leaves	0	36.7 ± 16.4	52.2 ± 11.1	11.1 ± 11.1	0	0	5.6 ± 3.7 b	7.8 ± 0.4

Values are mean ± SEM (%) of three replicates. ANOVA, Tukey's multiple range test. A different letter indicates a significant difference at $P < 0.05$.

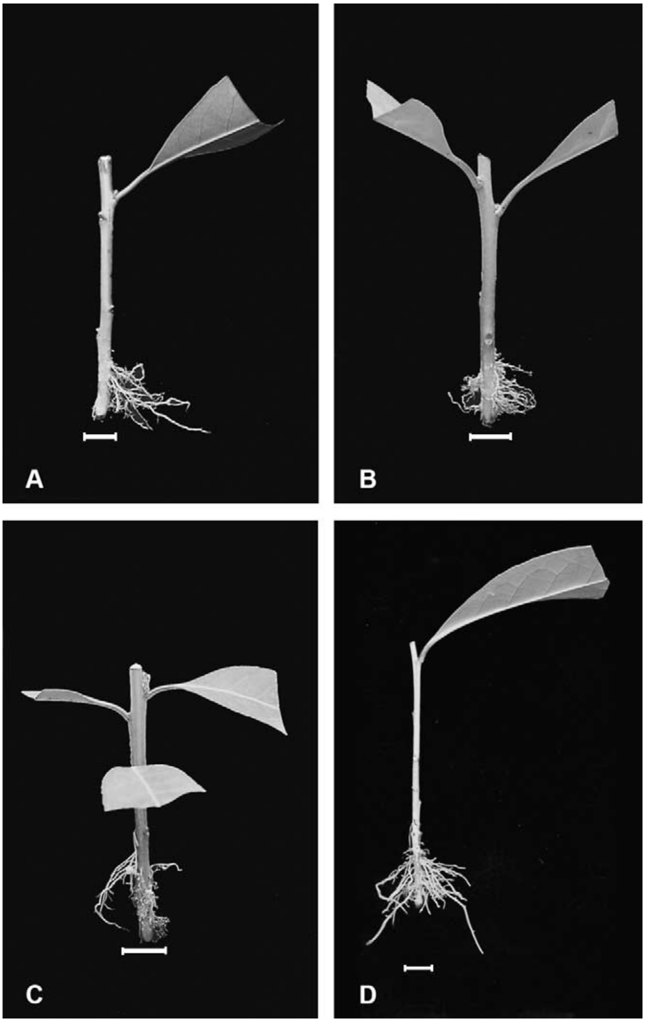

Fig. 3. Pattern of adventitious roots distributions in cuttings harvested from adult plants prepared either with one (A), two (B) and four (C) leaves or one leaf plus quercetine 30 μM (D). Note that *I. paraguariensis* presents a direct archetype of adventitious root formation without callus formation. Bar indicate 1 cm.

A strong relationship between the position of the leaf and the site of roots differentiation was observed. The adventitious roots were always originated at the base of the cuttings on the same orthostichy of the mature leaf. In fact, 100% of the rooted cuttings with a single leaf only formed roots along the leaf axis at the base of the cutting. Consequently, the distribution of roots was improved by increasing the number of leaves per cutting (Fig. 3A–C). Likewise, the mean number of first-order roots per rooted cuttings was improved by using cuttings with two to four attached leaves, even though they did not retain all of their leaves (Table 1).

Table 2
Effect of naringenin, quercetin and rutin on adventitious rooting of cuttings with one unfolded leaf harvested from 3- and 20-year-old plants

Treatment	Rooting (%)		Number of roots per rooted cutting	
	Juvenile (3-year old)	Adult (20-year old)	Juvenile (3-year old)	Adult (20-year old)
Control	91 ± 5.2 a	17 ± 3.9 a	10 ± 2.0 a	10 ± 3.2 a
Naringenin	90 ± 2.9 a	30 ± 2.0 ab	14 ± 2.0 a	10 ± 2.0 a
Quercetin	87 ± 7.5 a	55 ± 6.9 b	12 ± 2.7 a	15 ± 3.6 a
Rutin	88 ± 2.5 a	20 ± 5.8 a	19 ± 2.5 a	5 ± 1.7 a

Values are mean (±SEM) of three replicates. ANOVA, Tukey's multiple range test. A different letter indicates significant difference at $P \leq 0.05$.

3.2. *Effects of different flavonoids on adventitious rooting*

Adventitious rooting of adult material (20-year-old plants) varied from 17 to 55% and quercetin was significantly more effective than the other at promoting rooting (Table 2) treatments. However, statistical differences were not observed in cuttings from juvenile plants treated with flavonoids where, in general, the mean rooting was nearly 100%.

Likewise, the root number per rooted cutting was differentially affected by the flavonoids treatment (Table 2). Naringenin and rutin increased the roots number in juvenile material and decreased in adult material while quercetin notably promoted the regeneration of roots in cuttings from adult plants. The distribution of roots was improved by all tested flavonoids. Adventitious roots were regenerated around the cuttings and showed a similar pattern as a cutting with two or more unfolded leaves (Fig. 3D).

All cuttings without a single mature leaf died on account of an early browning and this process was not reverted by any treatment.

Adult plants showed higher content of rutin in the leaves than juvenile plants, while naringenin and quercetin were not detected at a detection limit of 0.2 mg l^{-1} (data not shown).

4. Discussion and conclusion

The results clearly show that retention of leaves on *Ilex paraguariensis* cuttings is essential for rooting. We observed that cuttings which shed their leaves, died afterwards. Concomitantly, we found a strong relationship between rooting and the retention of leaves throughout the course of incubation. The histological analysis showed anatomical evidences that the elapsed time for root formation was associated with leaf retention and in view of the fact that the adventitious roots were always originated at the base of the cuttings on the same ortostichy to which the leaflet belonged, the experiments, therefore, seem to prove that axial polarity in the adventitious root regeneration of the stem.

Our work also demonstrates that flavonoids affect the rooting process in *I. paraguariensis*. We found that quercetin promoted the formation of adventitious roots on cuttings from 20-year-old plants and all of them changed the distribution of roots around the cuttings without impacting the number of roots per rooted cutting. The influence of leaves on adventitious root initiation and expression phases has been widely interpreted in terms of a supply of auxin and nutritional factors (Jarvis, 1986; Gaspar et al., 1997 and literature cited therein). In addition many workers have implicated other specific factors in root formation and some have suggested that such factors may arise in the leaves, stem or buds (Blakesley, 1994; Haissig, 1986; Wilson and Van Staden, 1990). Several studies on woody plants have linked high endogenous flavonoids levels with ease of rooting (Hand, 1994 and literature cited therein) and later, Curir et al. (1990) have found that the accumulation of two endogenous flavonoids (identified as quercetin glycosides) increased the sensitivity of *Eucalyptus gunnii* microcuttings to the rooting stimulus exerted by the auxin.

In conclusion, this paper demonstrates that the leaf has some direct influence on the adventitious rooting of *I. paraguariensis* cuttings and the exogenous application of quercetin increases the rooting percentage and improves the distribution of roots around the cuttings from 20-year-old plants.

Acknowledgements

The authors are gratefull to Universidad Nacional del Nordeste (UNNE) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), for their financial support. We extend our deep appreciation to Establecimiento Las Marías and Establecimiento La Cachuera, for the award of fellowship to JT and CL. We thank to Alder Vásquez for preparing the illustrations and Prof. Martín Montiel Obregon for assistance in the preparation of English manuscript. We thank anonymous reviewers for their critical comments.

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