Inhibition Of Adventitious Rooting In *Backhousia Citriodora* F. Muell. Cuttings Correlate With The Concentration Of Essential Oil

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SUMMARY

Backhousia citriodora is typical of the many commercially valuable woody Australian Myrtaceae species that are recalcitrant in forming adventitious roots from cuttings after maturation. A series of experiments were conducted to identify an endogenous rooting inhibitor in line with established criteria. Endogenous levels of citral were correlated with the rooting capacities of juvenile versus mature, and easy- versus difficult-to-root genotypes of *B. citriodora*, in both winter and summer. The biological activity of citral was confirmed in bioassays on mung beans and easy-to-root *B. citriodora* seedlings. Evidence of a common mechanism of root inhibition with other species in the family Myrtaceae and the role of action of citral are discussed.

The inability to form adventitious roots from cuttings after maturation means many genotypes can only be propagated on a commercial scale from juvenile material (Curir *et al.*, 1993; Dhawan *et al.*, 1979; Paton and Willing, 1974). Included among these is *Backhousia citriodora* F. Muell., a tree or large shrub from which the commercially important essential oil citral is obtained. An embryonic plantation industry has begun to distil the oil for an expanding market. However, improvement of the industry is being held back by a need for selection and propagation of superior material that has both high oil quality and quantity (House *et al.*, 1996). Although easy- and difficult-to-root genotypes have been identified, even cuttings of the easy-to-root genotypes often remain unchanged for months before forming roots. Endemic to Queensland, Australia, *B. citriodora* belongs to the family Myrtaceae.

In a number of woody, Australian Myrtaceae species, failure to form adventitious roots has been correlated with endogenous rooting inhibitors. Paton *et al.* (1970) identified three endo peroxides in *Eucalyptus grandis* that inhibited the formation of adventitious roots from cuttings. Called the 'G factors' after *Eucalyptus grandis,* they were later found to be in other genera of the family (Dhawan *et al.* 1979). Researchers also identified similar endogenous rooting inhibitors in other woody members of the family Myrtaceae (Bolte *et al.* 1984; Crow *et al.*, 1977; Curir *et al.*, 1993; Paton and Willing, 1974).

Evidence for an association between endogenous substances and inhibition of rooting is usually based on a bioassay involving an easy-to-root species (Vieitez F. J. and Ballester, 1988; Vieitez J. *et al.*, 1987). However, Wilson and Van Staden (1991) cast doubt on the determination of rooting inhibitors and promoters using bioassays that involve easy-to-root mung beans. They demonstrated that 'G factors' and other solutes with no specific physiological activity can, depending on their concentration,

both promote and inhibit rooting of mung bean. Hackett (1988) proposed that the criteria for a true endogenous rooting inhibitor was:

The active substance must be identified.

A correlation must be established between endogenous levels of the identified substance and rooting capacity.

The extracted substance must be effective when applied to the species from which it was extracted.

The aim of this investigation was to identify endogenous compounds that account for the differences within *Backhousia citriodora* to form adventitious roots from cuttings. The specific objectives were to test the following hypotheses:

Genotypic differences in the ability to form adventitious roots from cuttings correlates with differences in the concentration of endogenous inhibitors.

The decrease in rooting ability once maturation occurs correlates with an increase in the concentration of endogenous inhibitors.

Identification of inhibitory compounds in *B. citriodora* will enable comparison with those in *E. grandis* to see if they are identical.

MATERIALS AND METHODS

A sequence of experiments was carried out to test the hypotheses and satisfy the criteria proposed by Hackett (1988) (Figure 1).

Collection of plant material.(1)

B. citriodora plant material was collected from numerous juvenile seedlings, each of three easy- and difficult-to-root genotypes, and a mature *E. grandis* tree. Tissue samples were taken of mature leaves, immature leaves and green semi-

hardwood stems of *B. citriodora* and mature leaves of *E. grandis*.

Extraction (2) and separation of compounds by descending chromatography (3)

Extractions were based on procedures of Vieitez and Ballester (1988) and Crowe W.D. (personal communication 2000). Fresh leaves were macerated in a blender and 1 g used for each sample. The samples were continuously extracted with methanol for 24 hours using a soxhlet apparatus in a darkened fume cabinet. The resulting aqueous residue was adjusted to a pH 3.5 with hydrogen chloride and partitioned 6 times with peroxide free ether. The ether extract was evaporated to dryness, redissolved in methanol (6 ml) and concentrated to 1 ml under nitrogen.

Concentrated samples were streaked onto filter paper (Whatman No. 3MM) at a rate of 1 ml for 23 cm width of paper. The chromatograms were equilibrated for 7 h and developed at room temperature by descending chromatography in isopropanol-ammonia-water (400:1:99). Development time was 14 hr and the distance run by the solvent fronts was close to 34 cm. Each R_F band was cut into three strips, each 7 cm long. Each strip of paper was used as a germination pad in a radish seed bioassay.

FIG. 1

Biological activity

The bioassays used to test for substances inhibiting root formation were *Raphanus sativus* (radish) seed germination (Paton *et al.*, 1970) and the rooting of *Vigna radiata* (L.) (mung bean) (Hess, 1959) and *B. citriodora* cuttings. In the radish seed germination bioassay each treatment of 20 radish seeds was replicated three times. To reduce dilution of the extract, minimal daily additions of water was added to the edges of the filter paper. Nicholls *et al.* (1970) reported that G inhibitors in *E.*

grandis were decomposed to inactive substances in water. Seeds were assessed as producing roots when the radicle was more than 3 mm long. A radish seed bioassay was carried out at each stage to confirm that inhibitors were still present and active.

The mung bean bioassay was adapted from Bassuk and Howard (1981). Two cuttings were placed in each of ten polycarbonate tubes with 5 ml of the test solution. The bases of the tubes were covered with aluminium-foil to keep light away from the area of root formation. Tubes were placed in a growth cabinet at 25°C with 12 hr light. Two ml of each solution was added to tubes every 2 days. Mung bean cuttings were treated with 5 ml of citral, nerol and geraniol in concentrations of 0, 1 x 10^{-1}, 10^{-2} 10^{-3}, 10^{-4} M.

B. citriodora cuttings were taken from easy-to-root seedlings at the 5-leaf stage, between the hypocotyl and the first leaves. Tips were removed down to the first developed leaves, which were cut in half. All other leaves were removed. Cuttings were placed in polycarbonate tubes filled with sieved perlite washed in distilled water. Cuttings were treated with 5 ml of citral, nerol or geraniol added in concentrations of 0, 1 x 10^{-2}, $10^{-3}$and 10^{-4} M. Two ml of each solution was added to tubes every 5 d.

Isolation and collection of compounds (4)

Concentrated samples were purified using a glass column (1.3 cm x 24 cm) filled with reverse phase silica R18 (55 µM – 105 µM. 125 Å). The silica was impregnated with the stationary phase of methanol and water (90:10) before packing the column. The column was eluted with methanol through a peristaltic pump running at 2 ml min-1 and fractions of the eluted extract were collected in 2 ml amounts. A bioassay using cuttings *B. citriodora* seedlings was used to confirm the presence of rooting inhibitors in the crude extract.

High performance liquid chromotogarphy analysis and correlation with rooting (4)

High performance liquid chromotogaphy (HPLC) analysis was conducted on extracts from easy-to-root and difficult-to-root genotypes and juvenile seedlings in June 2001 (winter) and February 2002 (summer), to identify differences in concentration between compounds that influence rooting

HPLC analysis was based on Vieitez and Ballester (1988) and L. Bauer (personal communication 2001). The extracted samples were dissolved in 6 ml of methanol and filtered through a Maxi-Clean™ Cartridge (0.2 μm, C^{18}, 600 mg). HPLC analysis was carried out under the following conditions: Injections (20 μl) were placed onto a Cosmil 5C18-MS column (4.6 cm x 15 cm) which was eluted with two solvent systems at a flow rate of 0.8 ml min^{-1} for 40 min. The solvent systems were A. methanol-water (32:68) and B. methanol-water (90:10). The water was acidified to pH 3.5 with acetic acid. The elution gradient was 7 min under an isocratic regime, solvent B 20% followed by 15 min under lineal gradient from 20% B to 100% B. UV detection was set at 240, 254 and 280 nm. An external standard of citral (100 μg ml-1) was injected after every six samples to standardise peak areas.

Compound concentrations were determined by peak area of the chromatograph. The presence of inhibitory compounds was monitored by radish seed bioassay, prior to HPLC analysis.

Identification of compounds (5)

Compounds were identified by comparison with authentic standards using gas chromatography-mass spectrometry (GCMS). Soxhlet extracted samples (methanol)

were placed under high vacuum (0.05 mmHg) with stirring at room temperature leaving a yellow-orange residue. The volatile material was collected in an in line liquid nitrogen trap. GCMS analysis was then conducted on the volatile and non-volatile components using an Alltech® GC column EC-5 (30 m x 0.25 mm internal diameter 0.25 μm) under the following conditions; the oven temperature was set at 100°C for 2 min then ramped at 16°C min-1 up to 270°C. Samples were injected at 200°C and the detector set at 280°C.

Statistical analysis

A logistic regression model, *genmod procedure* (SAS, 1998) was used to evaluate differences in germination between treatments in the radish seed bioassays. An overall test of significance used Chi-square analysis (SAS 1998). HPLC results were analysed using analysis of variance (SAS, 1998) to identify differences in concentrations of unknown compounds between plants of differing rooting abilities and between winter and summer. Analysis of variance (SAS, 1998) was also used in bioassays of mung bean cuttings and in cuttings from *B. citriodora* seedlings to compare the biological activity of endogenous compounds extracted from *B. citriodora*.

RESULTS

Bioassay of compounds separated by descending chromatography (3)

A seed bioassay of chromatograph strips showed that compounds with inhibitive activity were present in all tissue extracts. Germination was inhibited almost exclusively by extracts with an R_F value of 0.9. Compared with the control, germination was little affected by other R_F values. Accordingly results are

summarised for the R_F 0.9 band extracts (Table I). After 6 d only germination on extracts from the difficult-to-root genotype and *E. grandis* remained less than the control (Table I). Paton *et al.* (1970) reported inhibition at the same R_F value in a similar extraction and bioassay with *E. grandis*.

Table I

The chromatograph strips did not contain sufficient quantities of compound for use in bioassays involving cuttings. Although inhibitors were found in leaves and stems, subsequent extractions were obtained from mature leaves for ease of preparation.

B. citriodora cutting bioassay of the crude extract

By week eight, the crude extract from *B. citriodora* and *E. grandis* inhibited rooting of cuttings from easy-to-root genotypes of *B. citriodora* (Table Π).

Table Π

HPLC analysis of difficult- and easy-to-root forms (4)

Two compounds, with retention times of 27.6 and 29.3 min, were present in all extracts from *B. citriodora* but did not occur in samples from *E. grandis* (Figure 2). The concentrations of the endogenous compounds ranged from 45 µg g-1 fresh weight, in juvenile seedlings to 642 µg g-1 fresh weight in difficult-to-root genotype (Table ΙΙΙ).

TABLE ΙΙΙ

FIG. 2

The individual compounds were collected in 1 ml fractions, sealed and stored between 0 – 4°C for identification and testing. Re-analysis with HPLC showed that within 24 h the two isolated compounds had undergone isomerization resulting in both compounds being present in all fractions.

Identification of active compounds (5)

GCMS analysis of the volatile fraction showed only two components, identified as neral and geranial by comparison of mass spectra and retention times (4.75 min and 5.17 min respectively) against authentic standards. The mixture of *cis*-geranial and *trans*-neral are isomers of the essential oil citral. Co-injection of a mixture of the volatile fraction and authentic citral confirmed the identification of citral (Figure 3). The GCMS of the non-volatile fraction (*ca* 1% of the total material) revealed a gross mixture of unidentified substances, which had no significant effect on the germination of radish seeds. Germination of radish seeds treated with an extract of the non-volatile fraction was 90 % compared to 97 % germination in the distilled water control.

FIG. 3

Mung bean and B. citriodora cutting bioassays of citral and its isomeric derivatives (4)

In both the mung bean and *B. citriodora* bioassays, high concentrations of citral, nerol and geraniol inhibited rooting but were also toxic and low concentrations had little effect on rooting (Table IV). There were no differences in the biological activity between citral and its isomeric derivatives geraniol and nerol. In the mung bean bioassay cuttings in a concentration of 1×10^{-1} M were necrotic within 24 h.

After two days, stem bases in a concentration of 1 x 10^{-2} M citral and nerol were also necrotic. By day 4, stem bases in geraniol, at 1 x 10^{-2} M were chlorotic. Likewise in the *B. citriodora* bioassay, cuttings treated with geraniol and citral at 1 x 10^{-2} M were necrotic within seven days and cuttings treated with nerol at 1 x 10^{-2} M within 21 days. No toxicity was observed in cuttings placed in the lower concentrations of 1 x 10^{-3} M or 1 x 10^{-4} M. After nine days, the number of roots produced by each mung bean cutting in concentrations of 1 x 10^{-4} M geraniol, nerol and 1 x 10^{-3} M citral were higher than the control (Table IV). However, there was no difference in the number of roots between treatments of *B. citriodora*, with cuttings only forming one or two roots.

Table IV

At each stage of the procedure bioassays confirmed the presence of substances that inhibited radish seed germination.

DISCUSSION

The results support the hypothesis that a barrier to adventitious root formation on cuttings of *B. citriodora* is the presence of citral in the shoots from which cuttings are taken. A broad correlation was established between endogenous levels of citral and the rooting capacities of juvenile versus mature, and easy- versus difficult-to-root genotypes of *B. citriodora*, in both winter and summer.

The proposed causal role for citral is supported by the results of experiments following the criteria established by Hackett (1988). A putative inhibitor was isolated from *B. citriodora,* especially from that part of the shoot from which cuttings are taken. Its status as an inhibitor was repeatedly established using bioassays and its

specific inhibition of adventitious rooting of *B. citriodora* by application to easy-to root cuttings. A correlation was established between endogenous levels of inhibitor and the rooting capacities of juvenile, easy- and difficult-to-root genotypes. The inhibitor was then isolated and identified as citral, a mixture of neral and geranial by GCMS.

The findings are consistent with those of Chen (1997) who reported a negative correlation between essential oil yield and rooting success in *B. citriodora* cuttings taken from juvenile stock plants. Also consistent with the hypothesis is the report of House *et al.* (1996) that oil concentrations in seedlings of *B. citriodora* are generally lower than those of adult parent tree.

Citral was shown to be toxic to *B. citriodora* seedlings at concentrations slightly greater than endogenous levels. Conversion of endogenous citral concentrations to Molar values for 1 g of leaf fresh weight gives a Molar range from 3 x 10^{-4} M to 4 x 10^{-3} M (Table I). This falls between the applied concentrations of 'no effect' (1 x 10^{-4} M) and 'toxic effect' (1 x 10^{-2} M) (Table IV).

The results demonstrated that the inhibitory compounds in *B. citriodora* are similar but not identical to those in *E. grandis*. Inhibition of seed germination occurred at the same R_F value for extracts of both *B. citriodora* and *E. grandis* (Table I: Paton *et al.* 1970). The crude extract from *B. citriodora* and *E. grandis* inhibited rooting of cuttings from easy-to-root genotypes of *B. citriodora* (Table II). Thus the inhibitors present in *B. citriodora* and *E. grandis* can both act as specific rooting inhibitors in *B. citriodora*. However, isolation of inhibitors from *B. citriodora*, based on the methods of Baltas *et al.* (1992) and M. Baltas (personal communication 2001) for *E. grandis*, did not identify any active compounds. The procedure for isolating inhibitory compounds involved a number of stages. Radish seed bioassays confirmed

the presence of inhibitory compounds until the final stage of isolation when no compounds were detected (unpublished data). This suggests that citral is similar but not identical to the inhibitory 'G factors' in *E. grandis*.

To be effective as a rooting inhibitor, citral needs to be available at the site of rooting in high enough concentrations. Results from a seed germination bioassay indicate there are high concentrations of citral in the stem tissue (Table I) close to the site of root formation.

Not all the extracted citral would be involved in inhibition of rooting. Some of the endogenous citral would be isolated in oil glands. In addition, under typical propagation citral may be leached, metabolised or deactivated from the cutting over time. If true the concentration of citral shown to affect rooting may be higher than necessary. Leaching may explain the increase in rooting of seedlings treated with crude leaf extracts after 12 weeks (Table II). It is also consistent with the long time required by *B. citriodora* cuttings to form roots (unpublished data, Lake, 1998)

The propagation conditions may also affect the composition of citral and its inhibitory qualities. Citral is volatile and susceptible to chemical reaction, as observed in the formation of neral from geranial and *vice-versa*, during storage. Neral and geranial are respectively derived from the primary alcohols nerol and geraniol by oxidation, which have routinely been reported in the oil of *B. citriodora* (Brophy *et al.;* 1995; Doimo, 2001). The interaction, biological activity and abundance of neral, nerol, geranial and geraniol in *B. citriodora* suggest any of them may regulate the formation of adventitious roots (Table IV). This suggests that rooting inhibition is not dependent on the form of either neral or geranial.

Inconsistent with the proposed role of citral are the high concentrations of citral found in summer compared to winter. However, the critical concentration of

citral may be dependent on interactions with other factors associated with seasonal changes in the environment. There is evidence of such complex interactions between environmental factors and endogenous citral levels in other species (Ahmed *et al.*, 2001; Singh-Sangwan *et al.*, 1994)**.** The distribution of citral between the leaves and stem may also vary with environmental or other factors. Concentrations of citral at the site of root formation and its consequent inhibitory effect on rooting may differ from the concentration in the leaves (Ghisalberti, 1996). Alternatively, the sensitivity of tissue involved in root formation to citral may vary throughout the year.

The mode of action of citral as a rooting inhibitor is unknown. One theory is that citral is isolated in glands and is only released in response to wounding, when it inhibits rooting until it is leached from the medium. This is consistent with the theory that physical wounding of cuttings induces changes to cells, mediated by chemical promoters and inhibitors (Kodde and Van der Krieken, 2000; Wilson and Van Staden, 1990).

The increased number of mung bean roots at low concentrations of citral and inhibition of rooting at high concentrations (Table IV) is consistent with the findings of Wilson and Van Staden (1991) that irrespective of chemical identity, low concentrations promote rooting while high concentrations are inhibitory.

The findings of this research may apply to other woody Myrtaceae species. High concentrations of essential oils are common in Myrtaceae, especially the eucalypts (Boland *et al.*, 1991; Curir *et al.*, 1995), the majority of which are recalcitrant in forming roots from cuttings (Hartney, 1980; Paton *et al.*, 1970). In the difficult-to-root *Leptospermum petersonii* and *L. liversidgei*, neral and geranial were not found in seedlings but when seedlings had 15 nodes the two terpenes were found in concentrations that varied from 0 to > 50 % of the endogenous oil (Brophy *et al.*,

2000).

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TABLE I

Radish seed germination bioassay of paper chromatography strips of unknown compounds extracted from B. citriodora and E. grandis and having a R_F value of 0.9. Results are compared to a methanol-water control, after 2 and 6 days. Germination percentages with different letters are significantly different at P<0.05

Stock plant	Material source	Mean germination Day 2 (%)	Mean germination Day 6 (%)
E. grandis	Mature leaves	3 $_{c}$	20 $_{c}$
B. citriodora			
Difficult-to-root	Semi-hardwood stems	23 $_{bc}$	33 $_{bc}$
Difficult-to-root	Mature leaves	27 $_{bc}$	43 $_{bc}$
Difficult-to-root	Immature leaves	30 $_{bc}$	53 $_{ab}$
Easy-to-root	Semi-hardwood stems	30 $_{bc}$	50 $_{abc}$
Easy-to-root	Mature leaves	37 $_{b}$	50 $_{abc}$
Juvenile	Mature leaves	37 $_{b}$	60 $_{ab}$
Easy-to-root	Immature leaves	40 $_{b}$	80 $_{a}$
Control	-	73 $_{a}$	77 $_{a}$

TABLE II

Results of a bioassay to compare the effect of crude extracts from difficult-to-root genotype of B. citriodora and from E. grandis on the ability to inhibit rooting of cuttings from two easy-to-root genotypes of B. citriodora. Within weeks, treatments with different letters are significantly different at P<0.05

Treatment	Mean rooting percentage			
Extract	Week 8	Week 12	Week 16	Week 24
B. citriodora	50 $_{b}$	60	70	70
E. grandis	30 $_{b}$	50	50	50
Control	90 $_{a}$	90	90	90

TABLE III

Comparison of concentrations for compounds extracted from B. citriodora juvenile, from easy- and difficult-to-root genotypes during winter and summer. Means with different letters are significantly different at P<0.001

Unknown compound retention time (min)	Plant type	Season (µg/g FW of leaf)		Concentration mean (plant type)
		Winter	Summer	
RT.27	Difficult-to-root	291	603	447 $_{a}$
	Easy-to-root	189	559	374 $_{b}$
	Juvenile	45	506	277 $_{c}$
Seasonal mean		175 $_{y}$	556 $_{x}$	366
RT.29	Difficult-to-root	366	642	504 $_{a}$
	Easy-to-root	281	553	417 $_{b}$
	Juvenile	68	520	294 $_{c}$
Seasonal mean		238 $_{y}$	572 $_{x}$	405

TABLE IV

Bioassay of mung bean cuttings (after 9 days) and B. citriodora cuttings (after 4 weeks) to compare the biological activity of endogenous compounds extracted from B. citriodora on rooting. Treatments with different letters are significantly different at $P<0.05$

	Mung bean cuttings		B. citriodora cuttings
Treatment (Concentration M)	Rooted (%)	Number of roots	Rooted (%)
citral			
10^{-1}	0_{c}		-
10^{-2}	0_{c}		0_{b}
10^{-3}	60_{a}	6.8_{ab}	90_{a}
10^{-4}	95_{a}	6.8_{ab}	60_{a}
geraniol			
10^{-1}	0_{c}		-
10^{-2}	0_{c}		0_{b}
10^{-3}	35_{a}	5.7_{bc}	60_{a}
10^{-4}	75_{a}	8.5_{a}	70_{a}
Nerol			
10^{-1}	0_{c}		-
10^{-2}	0_{c}		0_{b}
10^{-3}	0_{c}		70_{a}
10^{-4}	85_{a}	5.1_{b}	80_{a}
Control	75_{a}	1.9_{c}	70_{a}

FIG. 1

Procedure for extraction of unknown compounds from *B. citriodora* adapted from Nicholls *et al.* (1970)

FIG. 2

Typical chromatographs of leaf extracts from (a) mature *E. grandis,* and from *B. citriodora* (b) difficult-to-root, (c) easy-to-root and (d) juvenile plants (UV = 240nm).

FIG. 3

GCMS analysis of (a) volatiles and citral standard, (b) authentic citral, (c) volatile fraction. GC column EC-5 (30 m x 0.25 mm internal diameter 0.25 μm). T (oven) 270 °C, T (injection) 200 °C, T (detector) 280 °C.

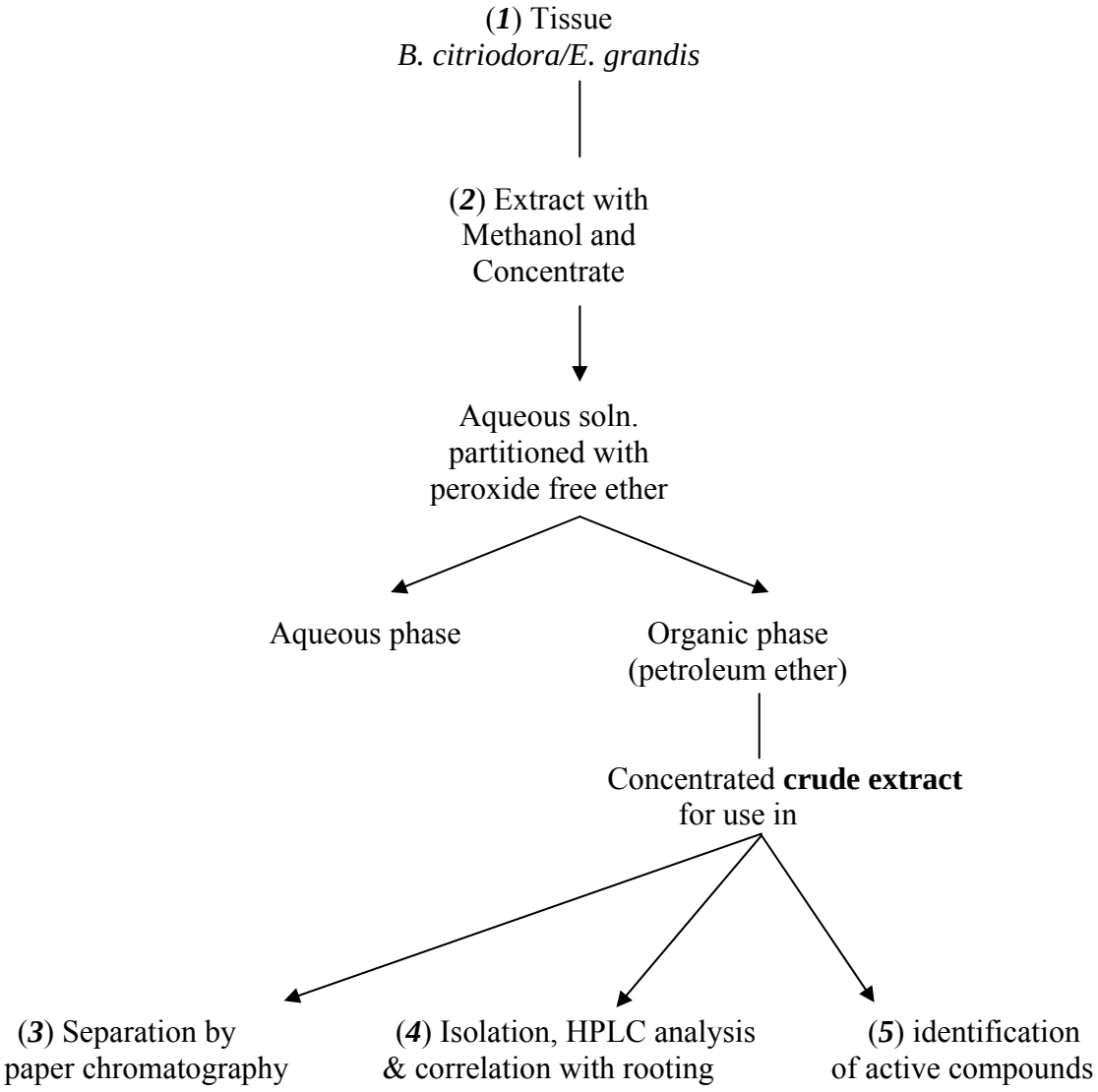
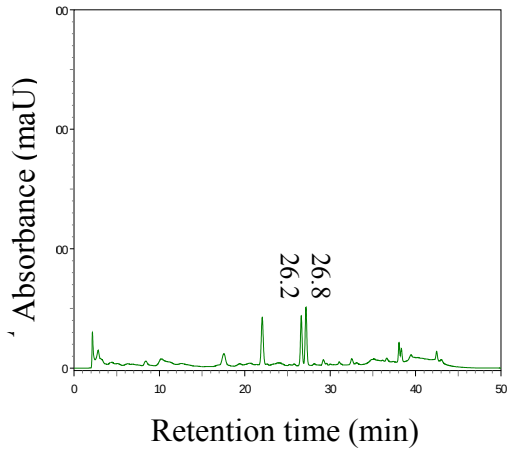
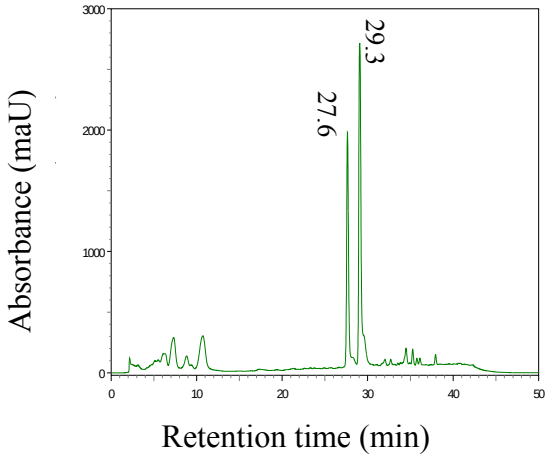

(1) Tissue
B. citriodora/E. grandis
(2) Extract with
Methanol and
Concentrate
Aqueous soln.
partitioned with
peroxide free ether
Aqueous phase
Organic phase
(petroleum ether)
Concentrated crude extract
for use in
(3) Separation by
paper chromatography
(4) Isolation, HPLC analysis
& correlation with rooting
(5) identification
of active compounds

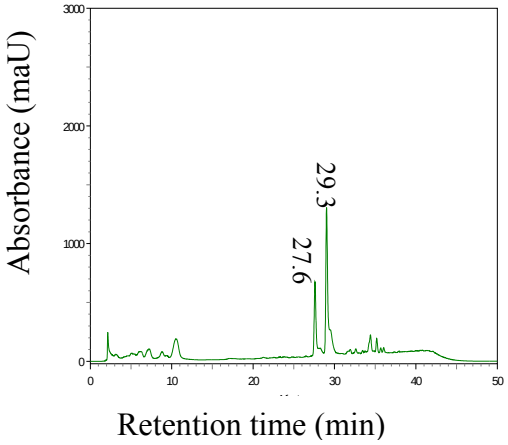
fig 1



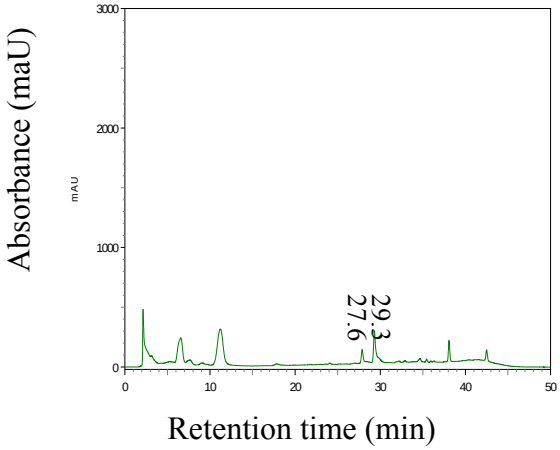
a. *Eucalyptus grandis* (mature)



b. *B. citriodora* (difficult-to-root)

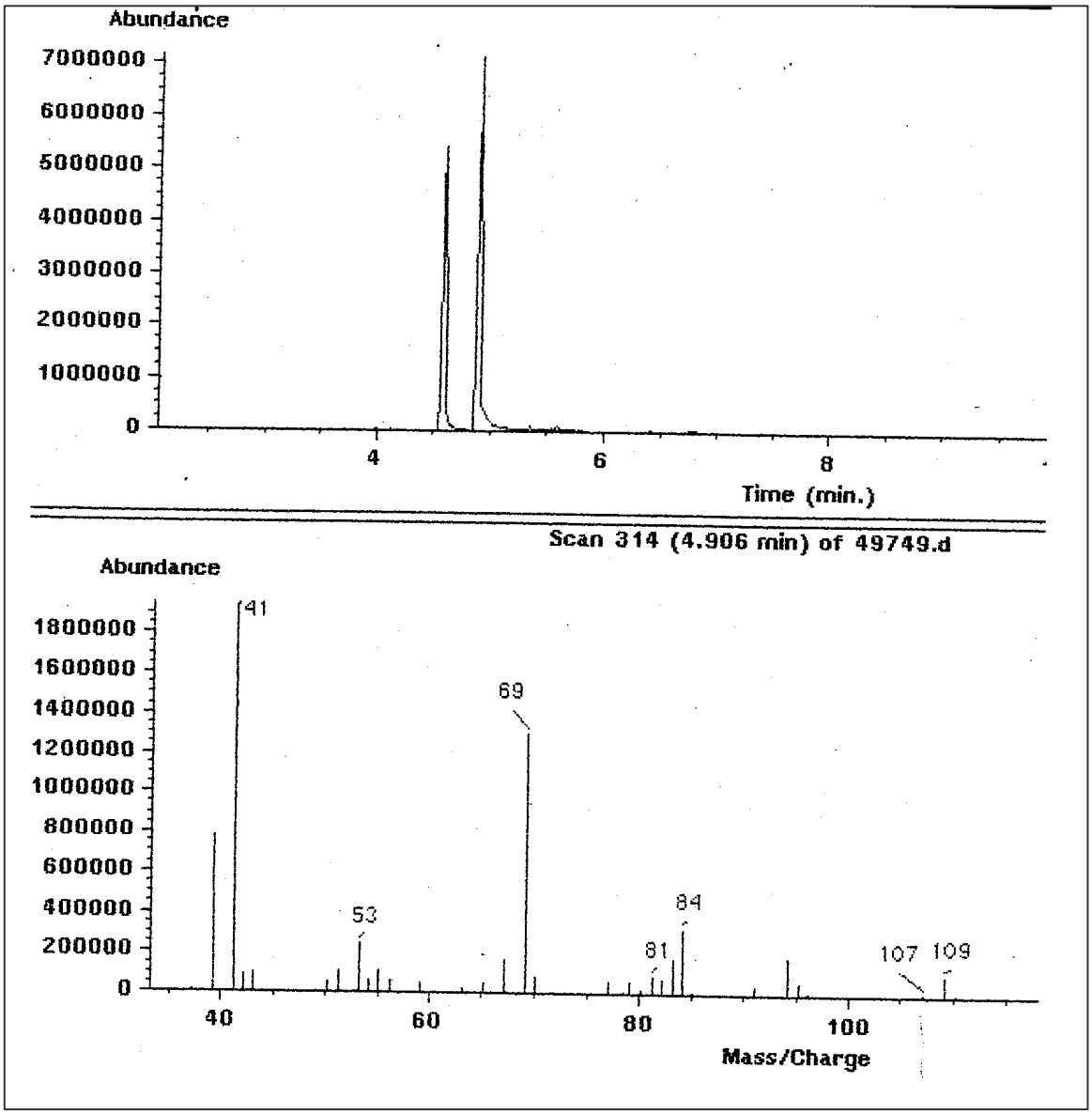


c. *B. citriodora* (easy-to-root)

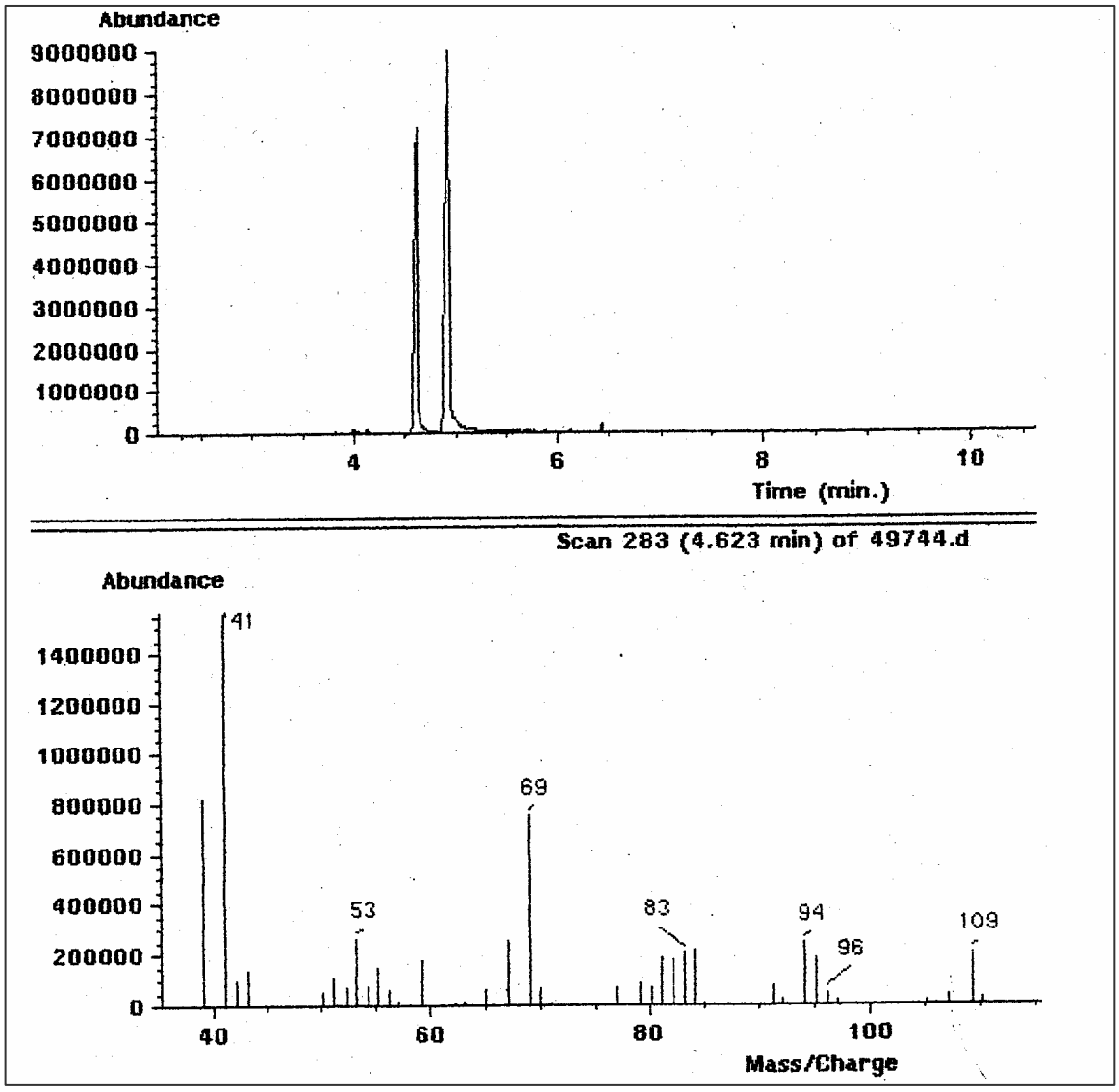


d. *B. citriodora* (juvenile material)

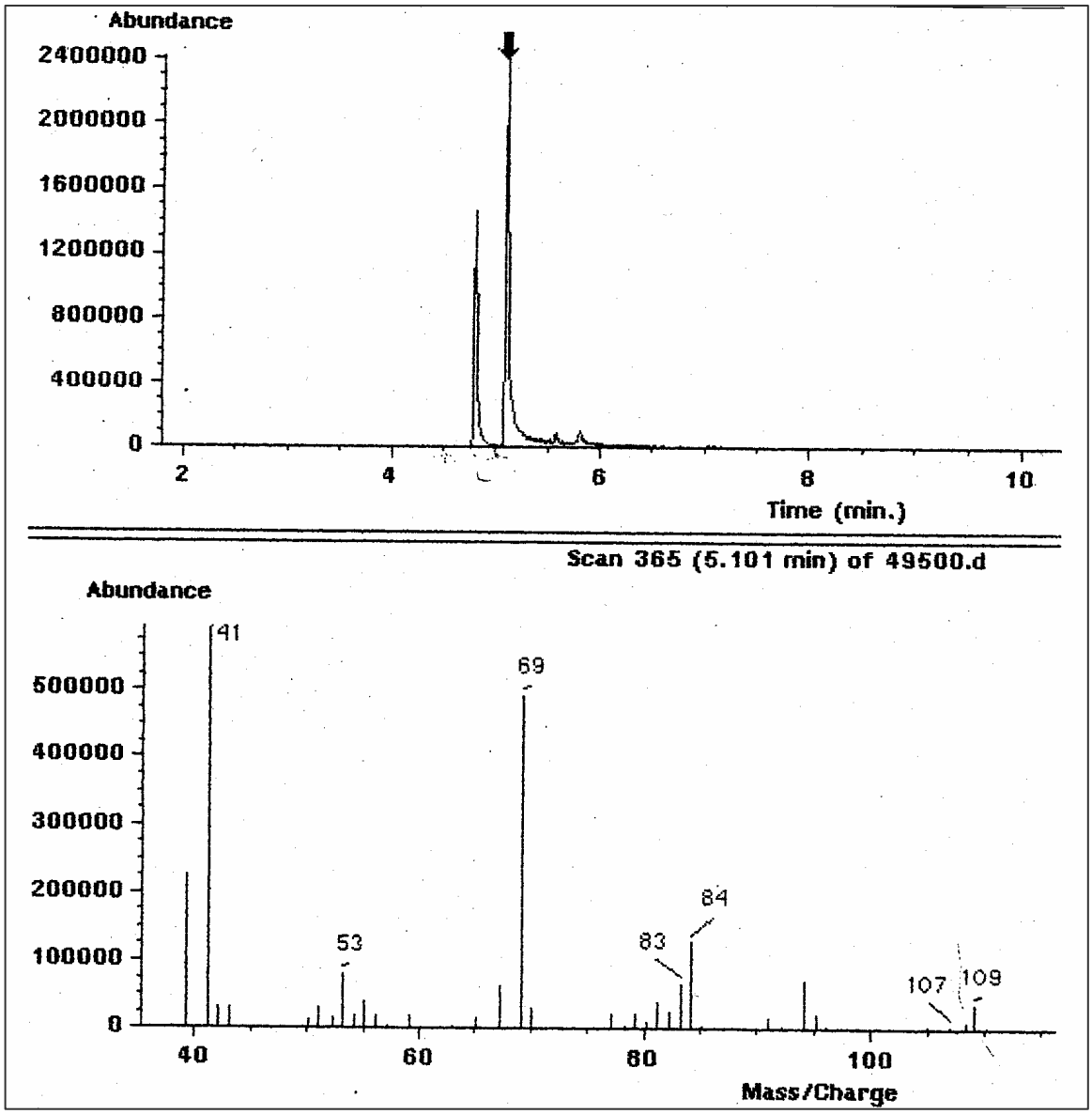
fig 2



a. volatiles and citral standard



b. authentic citral



c. volatile fraction

fig 3