CHAPTER 6

ALLELOPATHIC ACTIVITY OF PHENOLICS FROM *PROTEA CYNAROIDES* STEMS, AND THEIR ROLE AS ENDOGENOUS ROOTING REGULATORS *IN VIVO*

6.1 Abstract

Allelopathy bioassay indicated the presence of allelochemicals in *Protea cynaroides* stem cuttings. Further analysis of stem extracts identified large quantities of 3,4-dihydroxybenzoic acid and other similar phenolics in the stem. Phytotoxicity bioassay showed that 3,4-dihydroxybenzoic acid both stimulated and inhibited root growth of lettuce seedlings, depending on the concentration applied. The exogenous application of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants *in vitro* stimulated root growth at 100 mg l^{-1}, but not at lower concentrations, while root inhibition was observed at toxic levels (500 mg l^{-1}). HPLC analysis of cuttings during vegetative propagation showed a considerable increase in 3,4-dihydroxybenzoic acid levels from initial planting to when root formation took place, indicating that 3,4-dihydroxybenzoic acid may be an important phenolic compound in regulating root formation in *P. cynaroides* cuttings. HPLC analysis also identified caffeic, ferulic, gallic and salicylic acids in the cuttings.

6.2 Introduction

The quantification of total phenols in cuttings was reported in Chapter 5, which showed that the total phenol levels in *P. cynaroides* cuttings increased during root formation. In addition, the results showed that rooting took place when the total phenol content increased to more than three times the original level in untreated cuttings. Although very few studies have analyzed the phenolic contents of commercially important proteas, several phenolic compounds have been extracted and identified from *Protea rubropilosa* (Perold, Beylis and Howard, 1973), *Protea obtusifolia, Protea eximia* and *Protea neriifolia* (Verotta, Orsini, Pelizzoni, Torri and

Rogers, 1999). However, no research has been done on the effects of phenolic compounds on the rooting of proteas.

Phenolic compounds such as caffeic acid, ferulic acid, protocatechuic acid, *p*-hydrobenzoic acid and vanillic acid have all been identified as potential allelopathic agents (Einhellig, 2004). Dose-response data often show, depending on the concentration, both inhibitory and promotory effects on root growth of test species. Concentrations which exhibit root stimulation are usually a narrow band of doses at low levels, while root inhibition is found at higher concentrations.

In addition to the roles phenolic compounds play in allelopathy, they have also been identified as endogenous promoters and inhibitors of adventitious rooting. Their effects on the rooting of cuttings have been studied in numerous plant species, particularly in difficult-to-root plants. Various studies on this subject suggest that difficult-to-root stem cuttings tend to contain higher amounts of endogenous rooting inhibitors, which inhibits or delays root formation, compared to easy-to-root stems which have high promotory activity due to the presence of rooting promoters (Fadl and Hartmann, 1967; Richards, 1964; Taylor and Odon, 1970; Biran and Halevy, 1973; Reuveni and Adato, 1974). Furthermore, a number of known phenolic compounds such as catechol (Hackett, 1970), chlorogenic acid (Hammerschlag, 1982), phloroglucinol (James and Thurbon, 1981; Zimmerman, 1984) and phloretic acid (Jones and Hatfield, 1976) have been used to stimulate root formation, while rutin and tannic acid (Still, Dirr and Gartner, 1976) inhibit root formation in cuttings.

The poor rooting of protea cuttings has often been linked to them being hardwood plants, and therefore, inherently difficult to root. However, as mentioned above, because phenolics have been found to be important in the rooting of numerous other plant species, it is likely that phenolic compounds may indeed play a significant role as endogenous rooting regulators in *P. cynaroides*. The main objective of this study was to determine whether phenolic compounds found in *P. cynaroides* stems are the causal factors of rooting inhibition and/or stimulation. The phytotoxicity of aqueous stem extracts from *P. cynaroides* was assessed on lettuce seeds in a dose-response bioassay. Chemical analysis of compounds contained in stem extracts was done to establish the identity of phenolic compounds, and to determine if and to what extent

they may contribute to rooting inhibition and/or stimulation during *in vitro* and *in vivo* propagation.

6.3 Materials and methods

6.3.1 Collection of plant material

Plant material was obtained during autumn from *P. cynaroides* motherplants grown in an open field near Cullinan (25º40'32S; 28º31'20E; Altitude 1482 metres), situated in the Highveld region (summer rainfall) of South Africa. Semi-hardwood stems from the current season's growth, which were suitable to be used as cuttings, were removed from the motherplants and used for the bioassay studies, phenolic compound analyses and *in vivo* propagation.

6.3.2 Lettuce seed bioassay of stem extract

A dilution series of crude aqueous stem extract was prepared by soaking *P. cynaroides* stems in one-litre of distilled water for 24 hours in the dark at room temperature. Ten lettuce (*Lactuca sativa*) cv. 'Great Lakes' seeds were evenly spread on filter paper (Whatman No. 1) lining 9-cm Petri dishes. Five ml of the stem extracts, ranging from 200 to 5000 mg l^{-1}, were added to each Petri dish. Distilled water was used as the control. Each extract solution was replicated five times. After sealing the Petri dishes with Parafilm® to prevent moisture loss, they were placed in a growth chamber. The temperature of the growth chamber was kept at 25±2°C. Cool, white fluorescent tubes provided 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR) with a 12-hour photoperiod. After 6 days, the root lengths of germinated seedlings were measured and the mean root length from each treatment was calculated. A dose-response curve was drawn using the Curve-Fitting to Allelochemical Response Data (CARD) program (Liu, An, Johnson and Lovett, 2003).

6.3.3 Osmotic interference

The determination of osmotic potential of the extracts was done to exclude the possibility of osmotic inhibition in the bioassay, and to ensure that it is indeed the allelochemicals in the extract causing a reduction in the root growth of test species. The osmotic potentials of the stem extracts were measured with a Roebling digital micro-osmometer (Bothma, 2002).

6.3.4 Extraction, separation, isolation and characterization procedures

Fresh stems (100 g) of *P. cynaroides* were homogenized in 70% aqueous acetone for 6 hours. The extraction solution was then placed into a flask and dried under vacuum by using a Rotavapour (BUCHI® Rotavapor R-114) and a waterbath (BUCHI® Waterbath B-480). The fraction (2995 mg) was dissolved in water and submitted to a Diaion HP 20 chromatographic column (2.5 cm i.d. x 29 cm length). Elution was performed in a step-wise gradient of water:MeOH (100:0, 90:10, 80:20, 70:30, 60:40, 50:50. 40:60, 30:70, 20:80, 90:10), collecting 24 fractions of 100 ml and monitoring them by UV spectrophotometry (Pharmacia LKB Ultrospec III spectrophotometer) at 280 nm. A chromatogram was built from this data (Figure 6.1) and those fractions giving a chromatographic peak were pooled and concentrated in vacuum.

The combined fraction of the main peak (Fractions 16 – 20) was sent to the Natural Products Utilization Research Unit, USDA, USA, for separation and purification. Peaks were separated by high performance flash chromatography (HPFC) on a Horizon High Performance Flash Chromatography system (Biotage, Inc., Charlottesville, VA), using a reversed phase (12+ M C18, Biotage) column with the solvent gradient program set from 5% methanol to 45% methanol over 48 tubes (3 ml per tube), and finally washing the column with 100% methanol. The fractions were spotted on a reversed phase thin layer chromatography (TLC) plate (RP-18 F_{254s}, 10 x 20 cm, EM Science, Gibbstown, NJ) with developing solvent 20% methanol : 80% water. Spots were detected by observing under UV at 254 nm. Fractions with similar spots were combined into four semi-crude fractions: Fractions 1, 2, 3 and 4. Fraction 2 was further subjected to purification on RP-18 plate using developing solvent 15%

methanol:85% water. Four bands were collected from the plate. From these four bands, the one that was in the largest quantity, named Compound 1 (9.4 mg), was subjected to high performance liquid chromatography (HPLC) to confirm its purification, after which it was analyzed in the nuclear magnetic resonance (NMR) and mass spectrophotometer (MS) for characterization.

NMR data

The compound was dissolved in 0.5 ml of CD3OD. The proton spectra were acquired on a 200MHz Varian Mercury PLUS spectrometer at 199.97 MHz and Carbon 13 on 50.28 MHz.

MS data

The molecular mass of the compound was determined on a Shimadzu 2010EV spectrometer. For ionization, an atmospheric pressure chemical ionisation probe was used. The sample was dissolved in methanol and directly injected into the probe.

6.3.5 Lettuce seed bioassay of 3,4-dihydroxybenzoic acid

3,4-Dihydroxybenzoic acid was purchased from Merck®. A dilution series was prepared, ranging from 1 to 500 mg l^{-1}. Lettuce (*L. sativa*) cv. 'Great Lakes' was used as test species. The experimental design and growth conditions were the same as described in section 6.3.2. A dose-response curve was generated from the data using the CARD program (Liu *et al.*, 2003).

6.3.6 *In vitro* bioassay of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants

A concentration range similar to the one used in the allelopathic bioassay above (6.3.5) was incorporated into Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) to determine the effect of 3,4-dihydroxybenzoic acid on *in vitro* root growth of *P. cynaroides* seedlings. The MS medium consisted of sucrose (3%), 3 g l^{-1} Gelrite® and the respective concentrations of 3,4-dihydroxybenzoic acid. The pH of the medium was adjusted to 5.7 before autoclaving. In order to determine whether

3,4-dihydroxybenzoic acid was degraded during the medium sterilization procedure (autoclaving), 3,4-dihydroxybenzoic acid was autoclaved and analyzed in the HPLC.

Thirty-day old *in vitro*-germinated seedlings (sterilization and excision method described in Chapter 2) had their radicles removed at the hypocotyl and placed in the growth medium containing the different concentrations of 3,4-dihydroxybenzoic acid. Twenty explants per treatment medium were used, which were cultured in a growth chamber under a 12-hour photoperiod illuminated with cool, white fluorescent tubes providing 60 µmol m^{-2} s^{-1} Photosynthetic Active Radiation (PAR). The temperature of the growth chamber was adjusted to 25±2ºC.

6.3.7 Analysis of phenolic compounds by HPLC

Phenolic compound analyses were done on semi-hardwood stems taken from the motherplant, which were suitable to be used as cuttings, and on those that had been planted in the mistbed and rooted after 90 and 120 days. Phenolic compounds were analyzed using a HPLC (Hewlett Packard Agilent 1100 series) with DAD detection (diode array detector, 280, 325, 340 nm). A Luna 3u C-18 (Phenomenex®) reverse phase column (250 mm by 4.6 mm) was used. A gradient elution was performed with water (pH 2.6 adjusted with H_3PO_4) and acetonitrile (ACN) as follows: 0 min, 7% ACN; 0 – 20 min, 20% ACN; 20 – 28 min, 23% ACN; 28 – 40 min, 27%, ACN; 40 – 45 min, 29%, ACN; 45 – 47 min, 33%, ACN. The flow rate was 1 ml min^{-1}, and the injection volume was 20 µl. Identification of the phenolic compounds was done by comparing their retention times and UV apex spectrum to those of standards (purchased from Sigma Chemical Company, USA), which included syringic, gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, ferulic, caffeic, and chlorogenic acids. The amount of each identified compound was expressed in µg mg^{-1} of dry sample.

6.3.8 Statistical analysis

In the *in vitro* study on the effects of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants' root length and root fresh mass, twenty uniform explants were used in each medium treatment. Tukey's Studentized test was used to compare treatment means.

Statistical analyses were done in the SAS program (SAS Institute Inc, 1996). ANOVA are shown in Tables C44 and Table C45, Appendix C.

6.4 Results

6.4.1 Osmotic potential of stem extracts

Results from the osmometer readings showed that the osmolalities of the aqueous stem extracts ranged from 0 to 11 mOsmol.kg^{-1}. Data from experiments with polyethylene glycol (PEG-6000) solutions of increasing osmolality showed that no osmotic interference of lettuce seed germination or root growth occurred up to 53 mOsmol.kg^{-1} (Bothma, 2002). Therefore, the results for the stem extract concentrations which were used in the lettuce seed bioassay are considered to be indicative of allelopathic effects, and not distorted by osmotic effects.

6.4.2 Bioassay of stem extract

Results shown in Figure 6.2 illustrate a typical dose-response curve where aqueous solutions prepared from the stem crude extracts were able to both promote and inhibit rooting, depending on the extract concentration. At lower concentrations (200 - 800 mg l^{-1}), lettuce root growth was stimulated, while at higher concentrations of between 1000 mg l^{-1} and 5000 mg l^{-1}, reduction in root growth was observed. At the 400 mg l^{-1} stem extract solution, stimulation of root growth was the highest, which was 28% higher than the control. Although total inhibition was not observed, strong root inhibition was evident at the highest concentration (5000 mg l^{-1}).

6.4.3 Extraction, separation, isolation and characterization

Compound 1 was identified as 3,4-Dihydroxybenzoic acid (Protocatechuic acid): APCI-MS *m/z*: 153 $[M - H]^-$; ^{1}H NMR (CD_3OD, 200MHz): 6.75 (H5, 1H, d, J = 8.0 Hz, H-5), 7.39 (H6, 1H, dd, J = 2.0, 8.0 Hz, H-6), 7.43 (H2 1H, d, J = 2.0 Hz, H-2); ^{13}C NMR (CD_3OD, 200MHz): 115.7 (C-5), 117.7 (C-2), 123.9 (C-6), 146.03 (C-4), 151.52 (C-3), 170.28 (C-1).

The NMR spectral analysis of 3,4-dihydroxybenzoic acid, and its chemical structure are shown in Figure 6.3 and Figure 6.4, respectively.

6.4.4 Bioassay of 3,4-dihydroxybenzoic acid

Figure 6.5 illustrates the response of lettuce root growth to a concentration series of 3,4-dihydroxybenzoic acid. The effects of 3,4-dihydroxybenzoic acid on the lettuce root growth were typical of an allelochemical, with stimulation at low concentrations and inhibition at high concentrations. The highest stimulation was recorded at 100 mg l^{-1}, where the mean root length was 23% longer than the control. The ED_{50} value of 3,4-dihydroxybenzoic acid for lettuce, which was calculated from the CARD program (dose-response curve), was 339.1 mg l^{-1}. The ED_{50} value represents the effective dosage of 3,4-dihydroxybenzoic acid to cause 50% inhibition in lettuce root growth. The sharp drop in the dose-response curve from the concentration of 200 mg l^{-1} onwards indicates the contrasting activity of root stimulation and inhibition within a relatively narrow concentration range of between 100 mg l^{-1} and 200 mg l^{-1} (Figure 6.5).

6.4.5 Effects of 3,4-dihydroxybenzoic acid on *P. cynaroides in vitro*

Results from the HPLC analysis showed that the concentration of 3,4-dihydroxybenzoic acid remained unchanged after autoclaving, which indicated that the compound was not affected by high temperatures during the sterilization procedure. Table 6.1 shows the effects of 3,4-dihydroxybenzoic acid on the rooting of rootless seedlings after 3 weeks in culture. High biological variation was noted in the *P. cynaroides* explants, where the rooting percentage was relatively low and inconsistent throughout the concentration range. The inherent variation of *P. cynaroides* may have partly contributed to the differences in response to particular treatments. The other factor which contributed to the low rooting percentage recorded was the exclusion of roots which were less than 3 mm in length. These roots were extremely thin and barely visible to the naked eye, and explants with these roots were not considered rooted.

Despite the low rooting percentages, the amount of rooting by the *P. cynaroides* explants was affected by the concentration of 3,4-dihydroxybenzoic acid (Table 6.1). The response of the explants to 3,4-dihydroxybenzoic acid at the lower concentration range between 1 and 50 mg l^{-1} is noteworthy in that non-observable effects were obtained, i.e., root growth was similar to the control in terms of mean root length and root mass. However, an increase in mean root length and root mass was noticeable from 75 mg l^{-1}, even though the mean root length and root mass was not significantly higher (Table 6.1). Of importance was that the roots which formed on explants cultured on medium supplemented with 75 and 100 mg l^{-1} of 3,4-dihydroxybenzoic acid, were visibly thicker and fleshier (Figure 6.6). This was particularly evident at the 100 mg l^{-1} concentration, which coupled with longer roots, resulted in significantly higher mean root mass. Coincidentally, at the concentration level in which root growth was stimulated (100 mg l^{-1}), root stimulation was also observed in the lettuce bioassay.

Although a few explants produced roots in the medium containing the highest concentration (500 mg l^{-1}) of 3,4-dihydroxybenzoic acid, it was clear that the concentration was toxic to the explants. Rapid browning of cut surfaces of the explants was observed immediately after planting, which soon spread to other parts of the explant (Figure 6.6). This browning effect is also often seen when explants containing high phenol contents are cultured *in vitro*, where large amounts of phenols are leached from the explants and inhibit the formation of roots and ultimately causes the death of the explant (Debergh and Read, 1991; George, 1993).

6.4.6 Analysis of phenolic compounds by HPLC

From the HPLC analysis, 3,4-dihydroxybenzoic acid, caffeic, ferulic, gallic and salicylic acids were identified (Figure 6.7). In the stems taken from the motherplant, 3,4-dihydroxybenzoic acid (12.2 µg g^{-1}), caffeic (11 µg g^{-1}) and salicylic acids (32 µg g^{-1}) were found in relatively low amounts, while traces of ferulic and gallic acids were also detected. However, in the analyses of the basal end of cuttings taken from the mistbed when rooting began (after 90 days), high quantities of 3,4-dihydroxybenzoic acid was found (180.2 µg g^{-1}), which remained at a similar level after 120 days (188

µg g^{-1}) (Figure 6.7). At the same time, the levels of caffeic acid (28.6 µg g^{-1}) and salicylic acid (16.8 µg g^{-1}) remained low during the same time period.

Furthermore, from the results of the *in vitro* propagation study (6.4.5) and this study, a link between the concentration levels of 3,4-dihydroxybenzoic acid and root formation can be made. The concentration level (100 mg l^{-1} = 100 ppm) of 3,4-dihydroxybenzoic acid at which root stimulation of the *P. cynaroides* explants was observed, corresponded with the amount of 3,4-dihydroxybenzoic acid (180.2 µg g^{-1} = 180.2 ppm) found in the stem cuttings during rooting in the mistbed. It can therefore be deduced that once the concentration level of 3,4-dihydroxybenzoic acid in cuttings reached 100 mg l^{-1} or more, root formation was stimulated. In the *in vitro* propagation study, although the exact concentration between 100 mg l^{-1} and 500 mg l^{-1} where root stimulation would have ceased and inhibition commenced is not known, nevertheless, from the results for cuttings in the mistbed, it can be assumed that up to at least 180 mg l^{-1}, root formation would still be stimulated.

6.5 Discussion

Numerous papers have reported that phenolic compounds are both promoters and inhibitors of root formation, based on results of dose-response bioassays (Haissig, 1974; Still, Dirr and Gartner, 1976; Kling and Meyer, 1983). Based on results from the initial lettuce seed bioassay, where *P. cynaroides* crude stem extracts were used, root stimulation and inhibition occurred (Figure 6.1). Through further analyses of *P. cynaroides* stems, the phenolic allelochemical 3,4-dihydroxybenzoic acid was identified. Its identification was confirmed by comparing NMR and MS data with those reported in literature (Sang, Lapsley, Jeong, Lachance, Ho and Rosen, 2002), as well as with a reference standard. In total, twenty-eight fractions were isolated, of which the majority was found in very small amounts. Furthermore, NMR data showed that several of these fractions contained compounds which were similar in structure, and therefore, were derivatives of 3,4-dihydroxybenzoic acid, indicating that phenolics found in the stems of *P. cynaroides* are made up mostly of 3,4-dihydroxybenzoic acid and related compounds.

3,4-Dihydroxybenzoic acid is a common and widespread allelopathic agent that is able to influence growth at various stages of plant development (Rice, 1984). 3,4-Dihydroxybenzoic acid and other phenolic compounds have also been isolated from plant species such as *Arctostaphylos glandulosa* (Chou and Muller, 1972), *Chrysanthemum morifolium* (Kil and Lee, 1987), *Pennisetum clandestinum* and *Cunninghamia lanceolata* (Chou, Hwang and Peng, 1987), *Rumex japonicus* (Elzaawely, Xuan and Tawata, 2005) and *Vulpia myuros* (An, Haig and Pratley, 2000). Allelopathic bioassays of phenolics in these reports showed phytotoxicity to root growth. In addition, other chemically-related compounds such as 2,5-dihydroxybenzoic acid have also been reported to affect the rooting of *Tilia americana* (Morsink and Smith, 1975). Furthermore, from the report by Bär, Pfeifer and Dettner (1997), 3,4-dihydroxybenzoic acid extracted from three *Kalanchoe* spp. had non-observable effects at lower concentrations on the root growth of *Kalanchoe* cuttings, while root growth was inhibited at higher levels.

With regard to ED_{50} value, it is known that differences in the sensitivity of receiving plant species are keys to the inherent phytotoxicity of allelochemicals (Einhellig, 2004). Although no research paper could be found that reported the ED_{50} value of 3,4-dihydroxybenzoic acid on lettuce seeds, dose-response bioassays of other phytotoxins revealed that lettuce was less sensitive than other test species. For instance, with parthenin, the ED_{50} value for lettuce roots was four times higher than the dose level necessary to give the same response on the most sensitive species *Ageratum conyzoides* (Belz, Reinhardt, Foxcroft and Hurle, 2006). Thus, the effective dosage of 3,4-dihydroxybenzoic acid can be even more clearly shown when test species of different sensitivities are used. Further studies in this regard are necessary to allow comparisons between the effective dosages of 3,4-dihydroxybenzoic acid and other phytotoxins.

Results of the *in vitro* study further illustrated the effects of 3,4-dihydroxybenzoic acid on the root growth of plant species. No observable effects on the rooting of *P. cynaroides* were found at the lower concentrations, while stimulation of root growth was apparent when the concentration reached 100 mg l^{-1}. Although the exact concentration range of root stimulation is not known, it is clear that at 500 mg l^{-1}, 3,4-dihydroxybenzoic acid was toxic to the *P. cynaroides* explants, which was

demonstrated by root inhibition and browning of explants. Few studies have used *in vitro* conditions to determine the effects of plant-extracted phenolics on root growth. Nevertheless, Mucciarelli, Scannerini, Gallino and Maffei (2000) reported that root growth of *Nicotiana tabacum* explants, which were cultured on MS medium containing 3,4-dihydroxybenzoic acid, was also stimulated at low concentrations, while root inhibition was observed at the higher concentration level. In addition, several other phenolic compounds such as phloretic acid, phloroglucinol and chlorogenic acid are often incorporated into growth media at specific concentrations to induce *in vitro* rooting of explants as a substitute to conventional rooting hormones. This further demonstrates the importance of phenolics compounds, in general, in the propagation of plants.

Considerable differences in the level of 3,4-dihydroxybenzoic acid were found between stems taken from the motherplant and the basal ends of rooted cuttings in the mistbed after 90 days and 120 days. This indicated that the endogenous levels of 3,4-dihydroxybenzoic acid of the cuttings increased after they were planted, and that this high level was maintained during the rooting period. Although no reports have shown changes in endogenous levels of 3,4-dihydroxybenzoic acid during rooting of cuttings, Pellissier (1994) reported that root formation was stimulated by higher concentrations of 3,4-dihydroxybenzoic acid in *Picea abies*. Furthermore, the presence of other phenolic compounds such as caffeic, ferulic, gallic and salicylic acids, which were detected by HPLC analysis, is noteworthy. It is likely that the presence of these phenolics in the cuttings played a role in promoting rooting, since numerous allelopathy research papers have also identified caffeic, ferulic and gallic acids together with 3,4-dihydrixybenzoic acid in plants such as *C. lanceolata* (Zhiqun, Haig, Silong and Sijie, 2002), *Phytolacca* spp. (Kim, Johnson and Lee, 2005) and *R. japonicus* (Elzaawely *et al*, 2005). Furthermore, in the *C. lanceolata* extracts, which contained 3,4-dihydrixybenzoic acid, ferulic acid and gallic acid, stimulation of root growth of test species at high concentrations was observed (Zhiqun *et al*, 2002). Moreover, salicylic acid was also found to be stimulatory to root initiation of *Phaseolus aureus* cuttings (Kling and Meyer, 1983). It can therefore be assumed that this group of phenolic compounds has an important role to play in regulating root formation, particularly in terms of stimulation.

It is well known that several compounds usually act together in controlling many functions of a plant, including root formation. However, based on the relatively large amounts of 3,4-dihydroxybenzoic acid found in the stem extract, and on the stimulatory effects on root growth in *P. cynaroides* explants *in vitro*, as well as on the high endogenous levels of the compound in cuttings during rooting in the mistbed, it is proposed that 3,4-dihydroxybenzoic acid could play a primary role in root formation, depending largely on its endogenous concentration.

6.6 Conclusion

The current findings showed that a crude aqueous extract containing phenolic compounds from *P. cynaroides* stem cuttings exhibited allelopathic activity against root growth of lettuce. Chemical analyses revealed the presence of 3,4-dihydroxybenzoic acid in these stem cuttings. In addition, results from the dose-response bioassay and the *in vitro* experiment showed that 3,4-dihydroxybenzoic acid could either promote or inhibit rooting depending on its concentration. Large increases of 3,4-dihydroxybenzoic acid levels in cuttings during vegetative propagation suggests that root formation might be dependent on its endogenous levels. It was established that, in terms of the amount of 3,4-dihydroxybenzoic acid required for root formation to be stimulated, root formation took place when relatively high concentration levels (100 – 180 ppm) were reached. The results of this study provide a better understanding of how phenolic compounds might affect the rooting of *P. cynaroides* cuttings. Further investigations are needed to determine the full extent of the influence of 3,4-dihydroxybenzoic acid and other phenolics on root formation.

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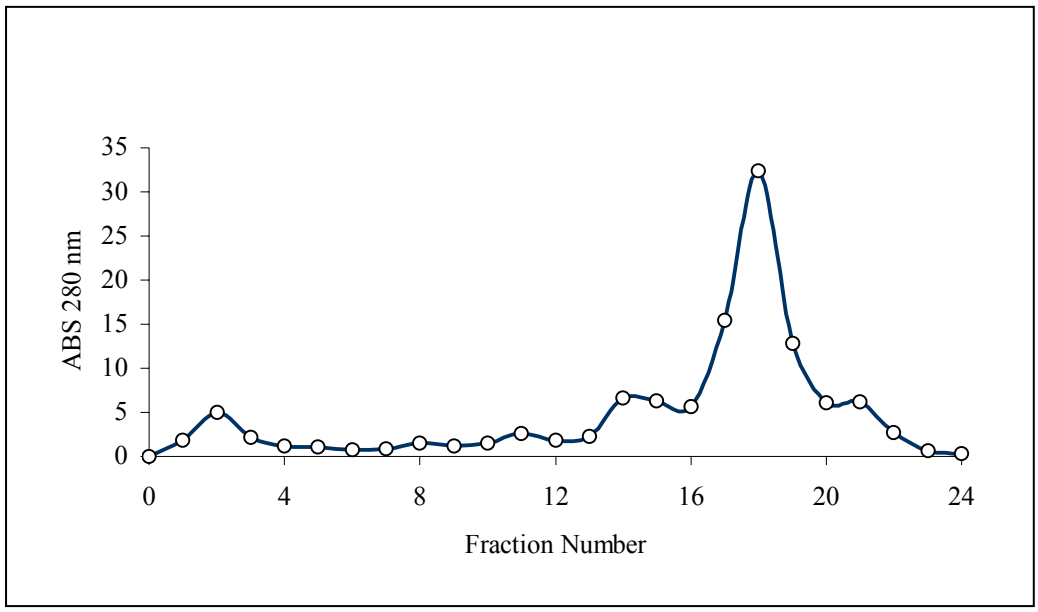

Figure 6.1. Column chromatography of 70% aqueous acetone extract from *P. cynaroides.*

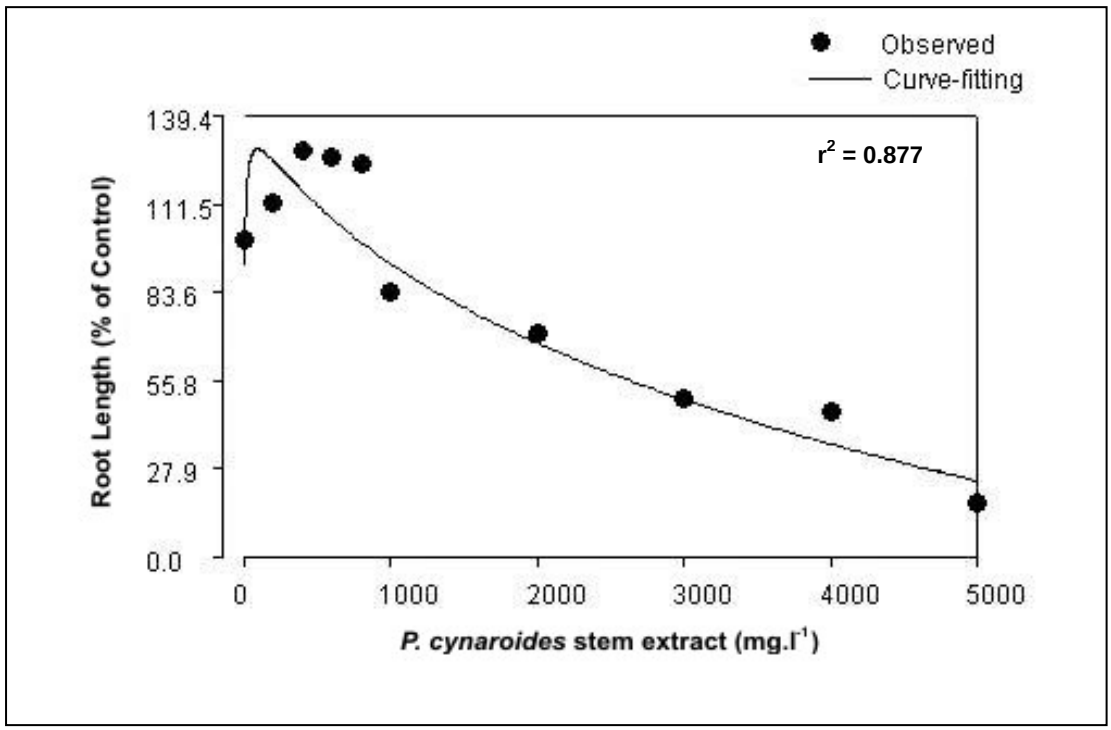

Figure 6.2. Dose-response of stem extract on lettuce root growth.

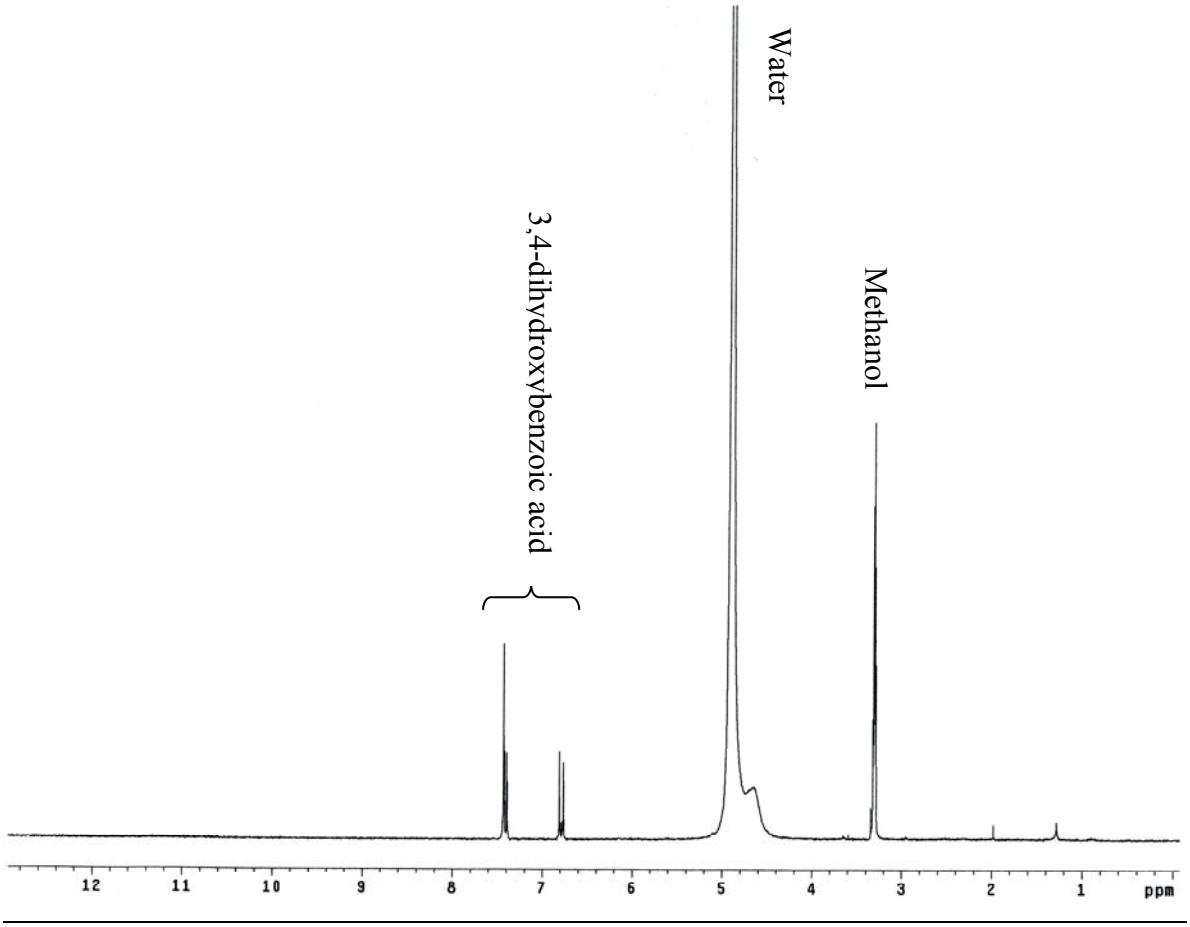

Figure 6.3. NMR spectral analysis of Compound 1 showing pure 3,4-dihydroxybenzoic acid.

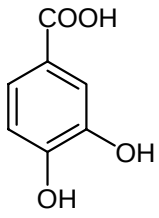

Figure 6.4. Chemical structure of 3,4-dihydroxybenzoic acid.

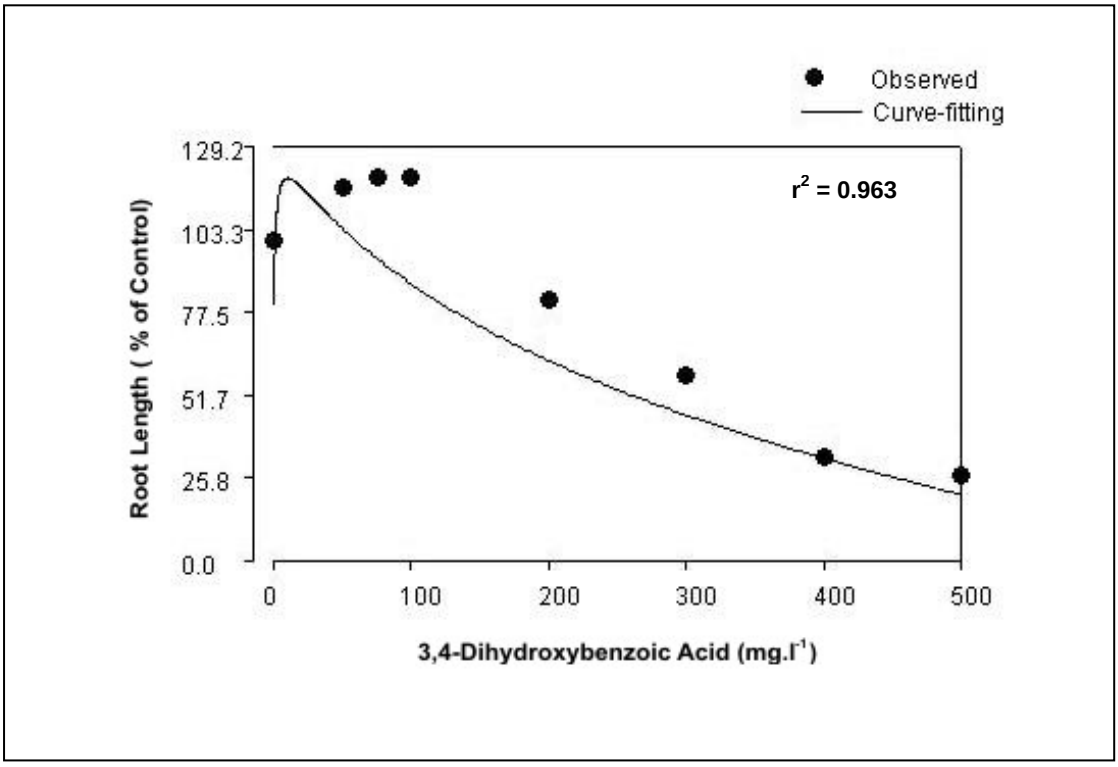

Figure 6.5. Dose-response of 3,4-dihydroxybenzoic acid on the root growth of lettuce seedlings.

Table 6.1. Response of *P. cynaroides* explants to a series of 3,4-dihydroxybenzoic acid concentrations on MS medium cultured *in vitro* (After 3 weeks in culture).

MS medium + 3,4-dihydroxybenzoic acid (mg l^{-1})	Rooting %	Mean root length (mm)	Mean root fresh mass (mg)
0	40	5.5 ± 0.93 ab	0.55 ± 0.17 b
1	40	5.25 ± 0.46 ab	0.58 ± 0.15 b
5	30	5.0 ± 0 ab	0.50 ± 0.06 b
25	10	5.0 ± 0 ab	0.55 ± 0.07 b
50	30	5.3 ± 0.52 ab	0.57 ± 0.08 b
75	20	5.75 ± 0.5 ab	0.88 ± 0.15 b
100	50	7.76 ± 2.79 a	2.66 ± 1.40 a
500	20	3 ± 0 b	0.3 ± 0.00 b

Means in each column followed by different letters are significantly different at $P \leq 0.05$ according to Tukey's studentised test.

Figure 6.6. Effect of 3,4-dihydroxybenzoic acid on *P. cynaroides* explants. From left to right: 0, 1, 25, 50, 100, 500 mg l^{-1} (After 3 weeks in culture).

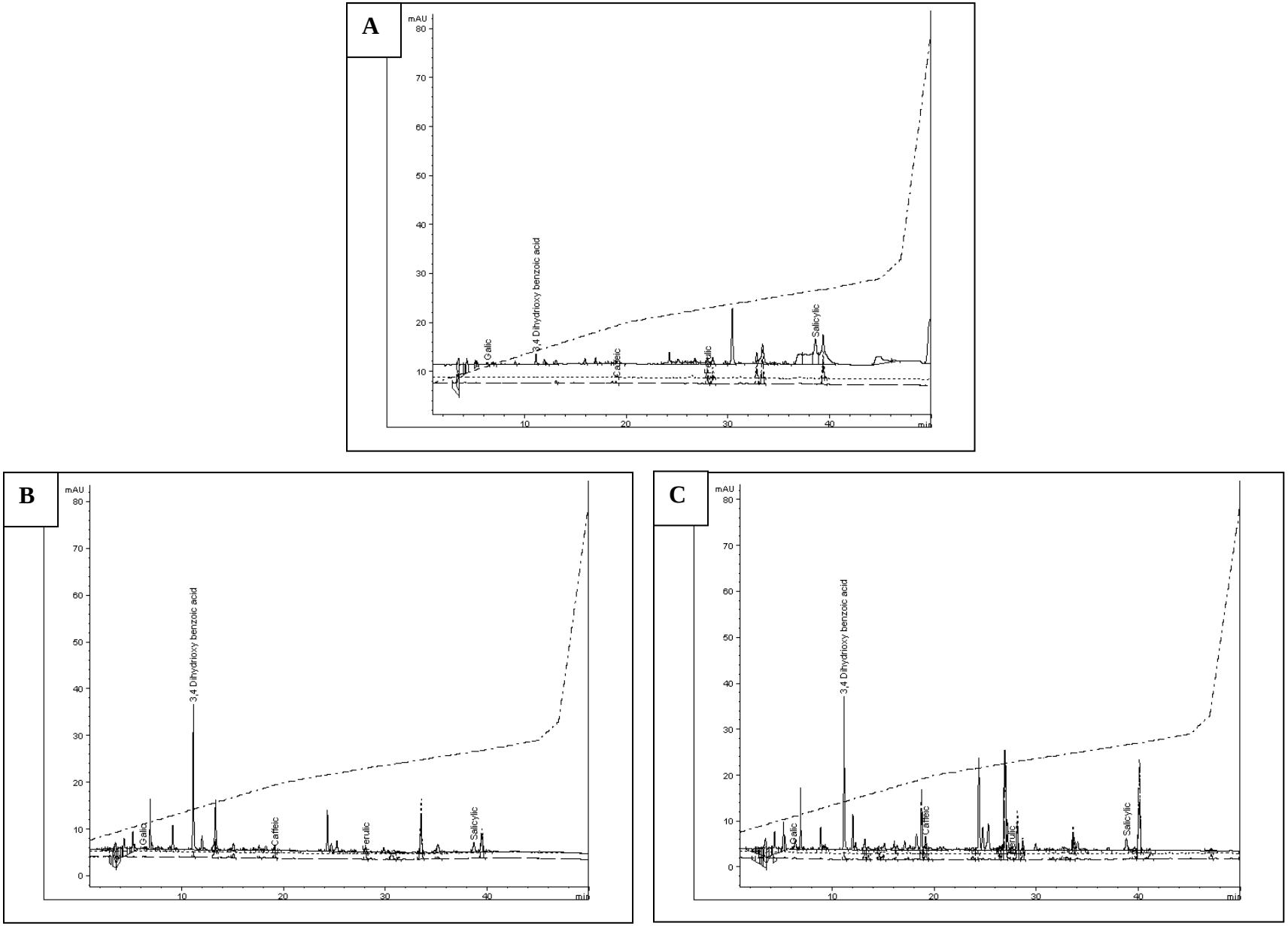

Figure 6.7. HPLC analysis of the basal end of stems. **(A)** Stems taken from motherplant; **(B)** After 90 days in mistbed when rooting was observed; **(C)** After 120 days when root growth was high.