Protein changes associated with adventitious root formation in hypocotyls of *Pinus radiata*

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Abstract

The changes in soluble proteins associated with adventitious root formation in hypocotyls of radiata pine (*Pinus radiata* D. Don) were studied using one- and two-dimensional polyacrylamide gel electrophoresis. Protein content decreased during the first day after root excision, and kept decreasing till the end of the time course under non-rooting conditions, *i.e.*, on medium without growth regulators, with indole-3-butyric acid (IBA) + kinetin, or with kinetin alone. During adventitious root initiation in response to IBA, however, the protein content began to increase from day 1 to its maximum at day 7, coinciding with the early stage of root initiation. A comparative analysis of protein changes by two-dimensional polyacrylamide gel electrophoresis showed 16 proteins that were probably associated with root initiation and development.

Additional key words: indole-3-butyric acid, kinetin, pine, protein patterns.

Introduction

It is well known that auxins play an important role in the regulation of root formation in cuttings (Haissig 1974, Jarvis 1986, Gaspar and Hofinger 1988). Auxin-induced adventitious root formation has been associated with an increase in the content of general soluble proteins (Kantharaj *et al.* 1979) and some specific proteins (Dhindsa *et al.* 1987). In addition to wound responses (Oliver *et al.* 1994) and other events including possible nutrient deficiency during rooting treatments or influence by microbes under non-axenic conditions, the increase of the proteins may be due to the alteration of gene expression during the root initiation (Dhindsa *et al.* 1987, Hand 1994, Kang 1995). Thus far, we are not aware of any information about the alteration of gene expression associated with adventitious root formation in radiata pine, an economically important species in which vegetative propagation plays an important part in commercial forestry in New Zealand (Reilly and Washer 1977). Furthermore, for the realization of the full potential of clonal forestry via stem cuttings from desirable clones which might be difficult to root, a better understanding of the molecular basis of the adventitious root formation is required. In this report, we examined the protein changes after radiata pine hypocotyl cuttings were de-rooted and then cultured in a nutrient medium with or without IBA, kinetin in combination with IBA or kinetin alone.

Materials and methods

Plants: Seeds were collected in 1995 from the same population of open-pollinated *Pinus radiata* D. Don trees grown in Canterbury, New Zealand. Seeds were surface-sterilized in 70 % (v/v) ethanol for 30 s, rinsed briefly in

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Abbreviations: 1D - one-dimensional; 2D - two-dimensional; EDTA - ethylenediaminetetraacetic acid; IBA - indole-3-butyric acid; IEF - isoelectric focusing; PAGE - polyacrylamide gel electrophoresis; PDA - piperazine diacrylamide; SDS - sodium dodecyl sulphate.

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sterile water, then soaked in 50 % (v/v) of a commercial bleach (containing 31.5 g dm^{-3} active sodium hypochlorite) for 30 min before being rinsed thoroughly with sterile water. The sterilized seeds were sown in autoclaved vermiculite in tissue culture jars, stratified in a cold room (temperature 4 °C) for one week, germinated in a warm dark room (26 °C) till seedlings emerged, and seedlings were transferred to a plant growth room (22 °C, continuous irradiance of 80 μmol m^{-2} s^{-1}). Uniform seedlings with hypocotyls that were 2.5 - 3 cm long and about 1 mm in diameter at the excision point (5 - 6 d after emergence) were selected to prepare cuttings. The root system was excised at about 2.5 cm below the cotyledonary node but was always above the hypocotyl and root junction. The cuttings were cultured in half-strength Murashige and Skoog (1962; MS) medium containing 2 % sucrose and supplemented with either 9 mg dm^{-3} IBA, or 10 mg dm^{-3} kinetin, or a combination of both (IBA + kinetin), or in medium without any phytohormones which served as a control. The lowermost 0.5 cm part of the cuttings were harvested at day 0, 1, 4, 7, 10, and 13 for all the analyses because adventitious roots were formed in this part. The harvested hypocotyl tissues were frozen in liquid nitrogen and stored at -80 °C.

Extraction of soluble proteins: Frozen hypocotyl tissues were ground to a fine powder using a mortar and pestle in liquid nitrogen. Then the powder was transferred to pre-chilled Eppendorf tubes. To run 1D SDS-PAGE, an extraction buffer [0.5 M Tris-HCl, pH 8.65, 50 mM EDTA, 0.1 M KCl and 2 % (v/v) 2-mercaptoethanol] used by Oliver *et al.* (1994) was added into the Eppendorf tuber at a ratio of 1:1 (m/v). The samples were intermittently vortex mixed for 5 min and then centrifuged at 17 400 *g* (*Eppendorf*™ *Centrifuge 5403*, Hamburg, Germany) at 4 °C for 15 min. The extracts for 2D-PAGE were prepared as described by Burritt (1992).

Determination of protein content: The soluble protein contents of the extracts for 1D SDS-PAGE were determined using the original Bradford method (Bradford 1976), while those for 2D PAGE were done using the modified Bradford method (Ramagli and Rodriguez 1985). This latter method reportedly enables an accurate estimation of the protein concentration of the extracts without interference by ampholyte and without precipitation of the protein. It was found necessary, however, to assay the protein content prior to the addition of protamine sulphate and urea to the extract, as both interfered even with this modified Bradford method. For this reason, aliquots of each extraction were maintained without adding these chemicals for the protein content to be assayed (Burritt 1992).

1D SDS-PAGE: Uniform 12 % T (m/v) gels were prepared according to Smith (1984) with the exception of piperazine diacrylamide (PDA) as a cross-linker instead of N,N'-methylene-bisacrylamide in order to reduce background staining of the gel matrix during silver staining (Hochstrasser *et al.* 1988b). A 2 × SDS sample buffer (Ausubel *et al.* 1989) was added at a ratio 1:4 (v/v) and then the sample was boiled for 5 min. After pouring electrode buffer (Hochstrasser *et al.* 1988a) into buffer chambers, the samples were loaded into the wells (approximate 4 μg protein per well) under the electrode buffer. Two gels were prepared and run together using the *PROTEAN*™ *II* chamber (*Bio-Rad*, Hercules, USA). Power was supplied by a *Model 3000xi* Computer Controlled Power Supply (*Bio-Rad*). Gels were run at constant current 50 mA during the stacking phase, after which the current was reduced to 30 mA.

2D PAGE: IEF separation was performed in the *PROTEAN*™ *II* chamber using the *PROTEAN*™ *II* tube gel adaptor. The *PROTEAN*™ *II* chamber was also employed for the second SDS-PAGE separation. IEF gel solution was prepared as described by Burritt (1992) based on Hochstrasser *et al.* (1988a). The anolyte contained 25 mM phosphoric acid and the catholyte contained 50 mM NaOH (Duncan and Hershey 1984). Sample containing approximately 20 μg protein was loaded on the top of each capillary tube and overlaid with 0.003 cm^3 of extraction buffer as above, and then the tube was filled with catholyte. Isoelectric focusing was performed at room temperature with the voltage increased in steps, *i.e.* 200 V 20 min, 400 V 20 min and 900 V 20 h (Mayer *et al.* 1987). Before removing the gels from the tubes, 0.001 cm^3 of concentrated bromophenol blue containing 50 % (v/v) aqueous glycerol saturated with bromophenol blue was loaded on the top of each gel. The dye marked the basic end of the gel and acted as a tracking dye in the second dimensional gel (Ausubel *et al.* 1989). The gels were removed, rinsed as described by Hochstrasser *et al.* (1988a) and Burritt (1992), sealed in Eppendorf tubes containing approximately 0.5 cm^3 transfer solution and immediately stored at -80 °C or transferred to SDS-PAGE gels without prior equilibration.

The 2D gels were similarly performed as 1D SDS-PAGE, but stacking gels were not employed. The IEF gels were immediately loaded on top of the second-dimensional gels after thawing out for 10 - 15 min at room temperature, and were not sealed with any agarose solution or filter paper and adhered well to the top of the second-dimensional gels (Burritt 1992). A piece of the protein molecular mass marker, which was prepared as described by Ausubel *et al.* (1989), was applied to the basic side of the IEF gel on the top of each second-

dimensional gel. The gels were run at 30 mA. No cooling was employed.

Silver staining was basically performed as described by Burritt (1992) with the exception that distilled water was used in ammoniacal silver nitrate solution instead of ethanol.

Comparison of 2D gels: Initially, 6 gels from day 0 (3 separate extractions, 2 gels for each extraction) were compared to establish a base pattern consisting of spots only present in all 3 extractions. Comparison of this base pattern to the patterns observed under various treatments was carried out in a pairwise-manner. All spots had been assigned a number for ease of reference and spots were only deemed to be differentially expressed if the difference was present in at least 2 of the 3 replicate extractions.

Results and discussion

Changes in protein content of IBA- and kinetin-treated cuttings: The protein content dropped in all treatments during the first day (Fig. 1). This pattern is similar to those obtained by Macisaac and Sawhney (1990) during auxin-stimulated and kinetin-inhibited lateral root initiation in *Lactuca sativa* roots. It has been suggested that this is possibly due to the uptake of water into the cuttings. The decrease continued till the end of time course (day 13) in control, IBA+kinetin and kinetin alone treatments. In contrast, the protein content in the IBA treatment increased to its maximum till day 7 before exhibiting a decline which might be associated with a substantial increase in the fresh mass of the tissues starting from this time (data not shown). The result clearly indicates that the early stages of the IBA-induced adventitious root initiation (day 4 - 7) in the cuttings of *Pinus radiata* hypocotyls are associated with an increase of soluble protein content. This was also demonstrated in *Lactuca sativa* lateral root initiation (Macisaac and Sawhney 1990) and in *Hydrangea macrophylla* adventitious root initiation (Molnar and LaCroix 1972).

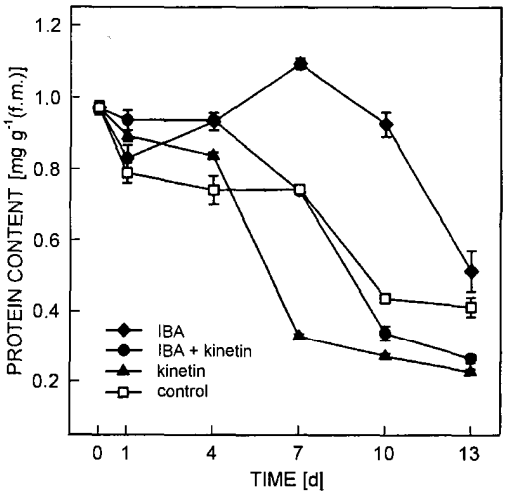

Fig. 1. Changes of protein content in the hypocotyl rooting region of radiata pine during rooting initiation. *Vertical bars* represent SE for 3 determinations from 3 individual extracts. Where no bars are shown they are smaller than the symbols.

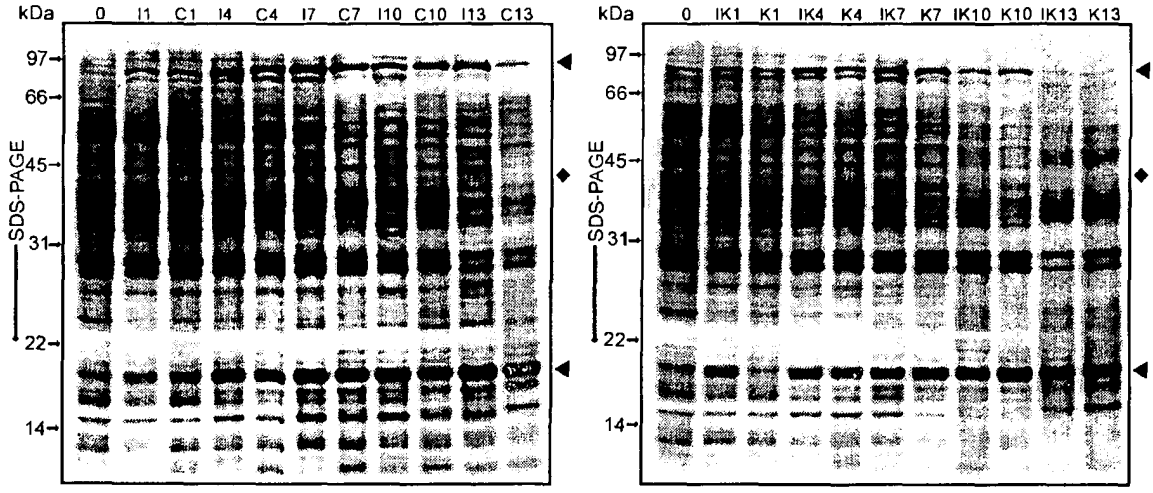

Fig. 2. Silver-stained SDS-PAGE of proteins extracted from rooting region in hypocotyls of radiata pine. The medium was supplemented by IBA (I1, I4, I7, I10, I13), growth regulator-free (C1, C4, C7, C10, C13), supplemented by IBA+kinetin (IK1, IK4, IK7, IK10, IK13), and kinetin alone (K1, K4, K7, K10, K13) from day 0 to 13. The numbers on the left indicate molecular mass. Bands disappeared after day 0 (*diamonds*), or increased in intensity in response to *in vitro* culture (*triangles*) are indicated.

Protein changes shown by 1D SDS-PAGE: There was a day 0 specific protein which decreased after the first day of culturing the hypocotyl cuttings in all 4 treatments, while there were two proteins which seemed to increase in abundance in response to *in vitro* conditions and not specific to any culture medium (Fig. 2). Overall, only few minor changes of protein pattern were detected among the 4 different treatments at the various times. One dimensional SDS-PAGE may not be suitable to detect the changes in protein patterns during adventitious root

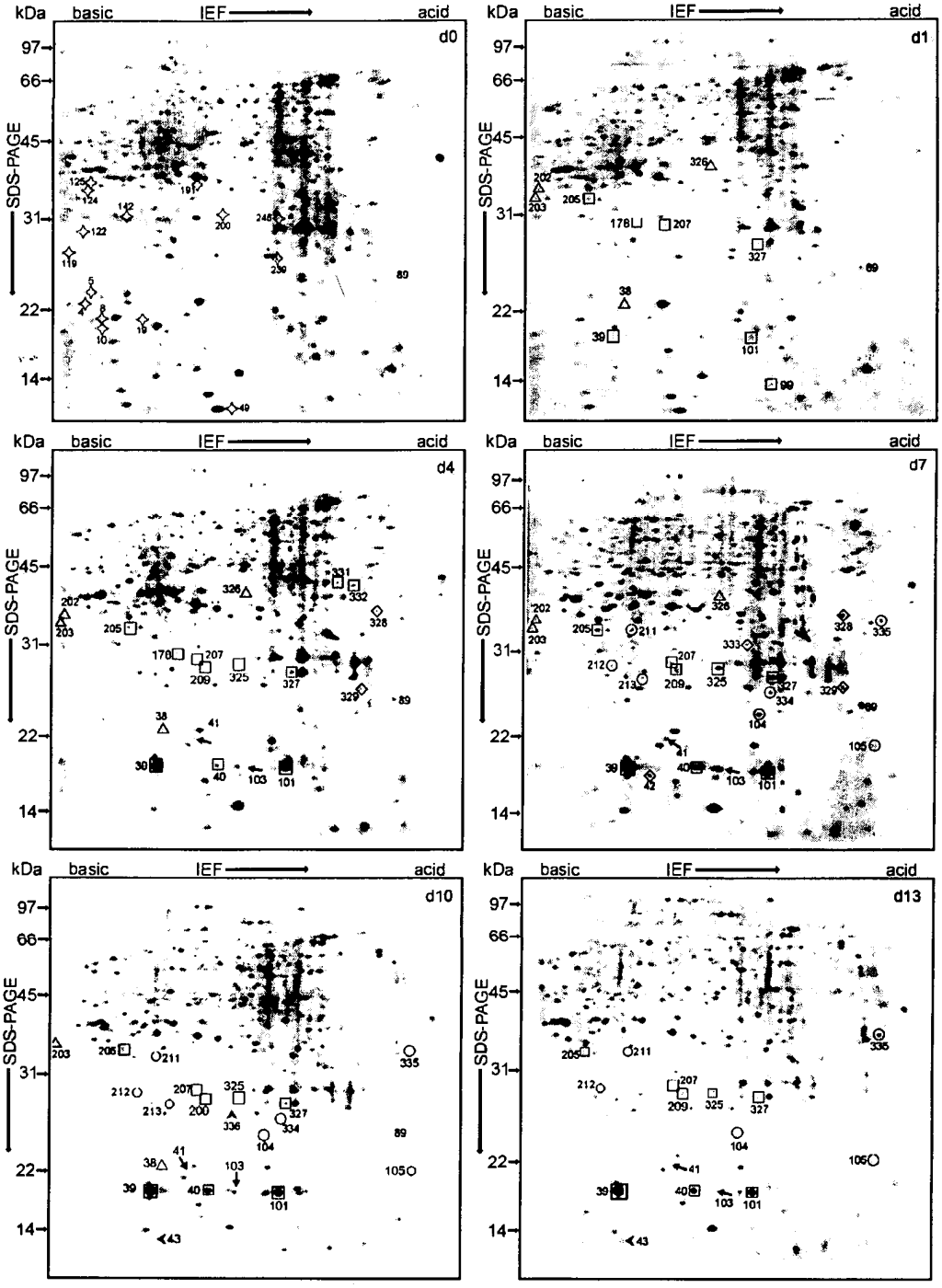

Fig. 3. Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of radiata pine treated with IBA at day 0, 1, 4, 7, 10 and 13. Proteins associated with wounding/tissue culture, induced by IBA preceding cell division associated with root initiation, root primordium initiation and formation, development of root primordium, root emergence only, and whole rooting process are indicated.

formation in some experimental systems, in which the changes in very few cells involved in rooting initiation could be "diluted" by the stable protein content in other cells. For example, Kang *et al.* (1995) did not detect any proteins associated with root formation in gladiolus callus by 1D SDS-PAGE. However, Macisaac and Sawhney (1990) successfully detected several proteins which were associated with lateral root formation in lettuce.

Changes in protein patterns shown by 2D PAGE: In order to gain a better insight into changes in gene expression associated with adventitious root formation in hypocotyls of radiata pine, comparisons between the patterns of protein extracted from hypocotyl cuttings treated with IBA (rooting) and without phytohormones (control) at particular times were made by 2D-PAGE. Approximately 600 protein spots were observed at day 0 when the cuttings were taken (Fig. 3). This pattern was considered the control pattern to which all other patterns were compared. Preliminary comparison indicated that major changes in the protein patterns were associated with those smaller than 45 kDa, probably because the resolution of proteins of higher molecular mass on 2D-PAGE is relatively poor (Dhindsa *et al.* 1987). Therefore, particular attention was paid to the 45 kDa region and below. Three hundred and thirty-six proteins were detected in this region.

The changes in protein patterns showed a varied spectrum and were divided into 7 major groups with several subgroups. Group 1 includes 108 proteins which were likely to be the products of basic metabolism or "housekeeping gene" products as they were detected at approximately constant levels throughout the time course in both treatments.

Group 2 including 134 proteins might be related to hypocotyl physiology and/or autotrophic growth because they disappeared or their levels decreased in both treatments. Forty-eight of the 134 proteins disappeared quickly during the initial period of culture. This may be one reason why protein content dropped at day 1 in all cases (Fig. 1). Dhindsa *et al.* (1987) also found that some protein spots considerably decreased or disappeared during 24 h-treatment with auxin, antiauxin and water (control). There was another group of 8 proteins which disappeared later (from day 7 thereafter). Meanwhile, the speed of disappearance of 18 proteins seemed to be related to IBA. The disappearance of 8 of these 18 proteins was slower in IBA-treated cuttings than in controls, while that of the other 10 was faster. The other 60 proteins in Group 2 disappeared irregularly at some stage and their behaviour was not clear.

Group 3 consisted of 34 proteins which were present at day 0 but disappeared only in IBA-treated plants. The disappearance of these proteins may be due to some unknown role played by IBA directly or indirectly. Group 4 consisted of 14 proteins which were present at day 0 and at IBA-treatment but disappeared in control. In group 5, 21 proteins were present in both treatments after excision of hypocotyls. These proteins were considered as a response to wounding and/or tissue culture. Nine of them appeared at some stages irregularly, while 12 appeared at all time from day 1 to 13 (Figs. 3, 4). In particular, three of them numbered 39, 40 and 101 increased markedly in abundance from day 4 and can be matched to the band with an apparent molecular mass of 17.8 kDa on 1D SDS-PAGE. Group 6 consisted of 6 proteins only detected in controls.

Searching for newly synthesized proteins which were related to root initiation and development is the major objective in this study. In the IBA treatment only, 19 newly appeared proteins, which were subsequently divided into 5 subgroups, were observed. In Subgroup 1, 4 proteins appeared from day 1 (Fig. 3) and preceded the first cell division which began at day 4 (Smith and Thorpe 1975, Li and Leung, unpublished). It remains to be determined if they had anything to do with root initiation or they might be induced by IBA. Subgroup 2 includes 4 proteins which appeared during days 4 to 7 (Fig. 3), coinciding with root primordium initiation and formation. There were 7 proteins in Subgroup 3 which appeared during days 7 to 13 and were likely to be associated with the later stages of root primordium formation and root development (Fig. 3). The fourth Subgroup includes 2 proteins which appeared during days 10 to 13 and might be associated with root development (Fig. 3). Some of these proteins in Subgroup 3 and 4 may be root-specific proteins and could be detected in root tissues (seedling and adventitious roots), particularly the protein numbered 335 in subgroup 3 increased in abundance correlating with root emergence. The last subgroup includes 2 proteins which appeared during the whole rooting process, i.e. day 4 - 13 (Fig. 3). One of the two proteins, numbered 103, appeared firstly at day 4, then increased in abundance till its maximum at day 7 and subsequently decreased, was particularly interesting.

Very similar to the study of Oliver *et al.* (1994), most of the differences in proteins between plants treated with IBA and control plants were of such small magnitude that it was difficult to quantify them and establish them as novel proteins. This does not diminish the possible importance of some of these changes. In fact, small changes in protein synthesis, for example enzymes, may cause large changes in developmental systems. While the technique of 2D-PAGE seems to be limited in its ability to consistently track such small alterations, it is clear that there was a complex pattern in changes in gene expression of the radiata pine hypocotyl in the IBA treatment and some of which could be associated with the various stages of root formation. Because our rooting experiments were carried out under axenic and nutrient

non-limiting conditions, it should be recognized that those changes in proteins proposed to be associated with the various stages of root formation were at least not caused by some possible spurious situations. Further study utilizing more powerful molecular techniques such as differential cDNA cloning and *in situ* hybridization is being undertaken to isolate putative cDNA clones specific to root initiation in this radiata pine system and to make comparison with auxin-induced cDNA clones from other adventitious rooting systems including mung bean hypocotyls (Chen *et al.* 1996), apple microcuttings (Butler and Gallagher 1999) and hypocotyl cuttings of loblolly pine (Hutchison *et al.* 1999).

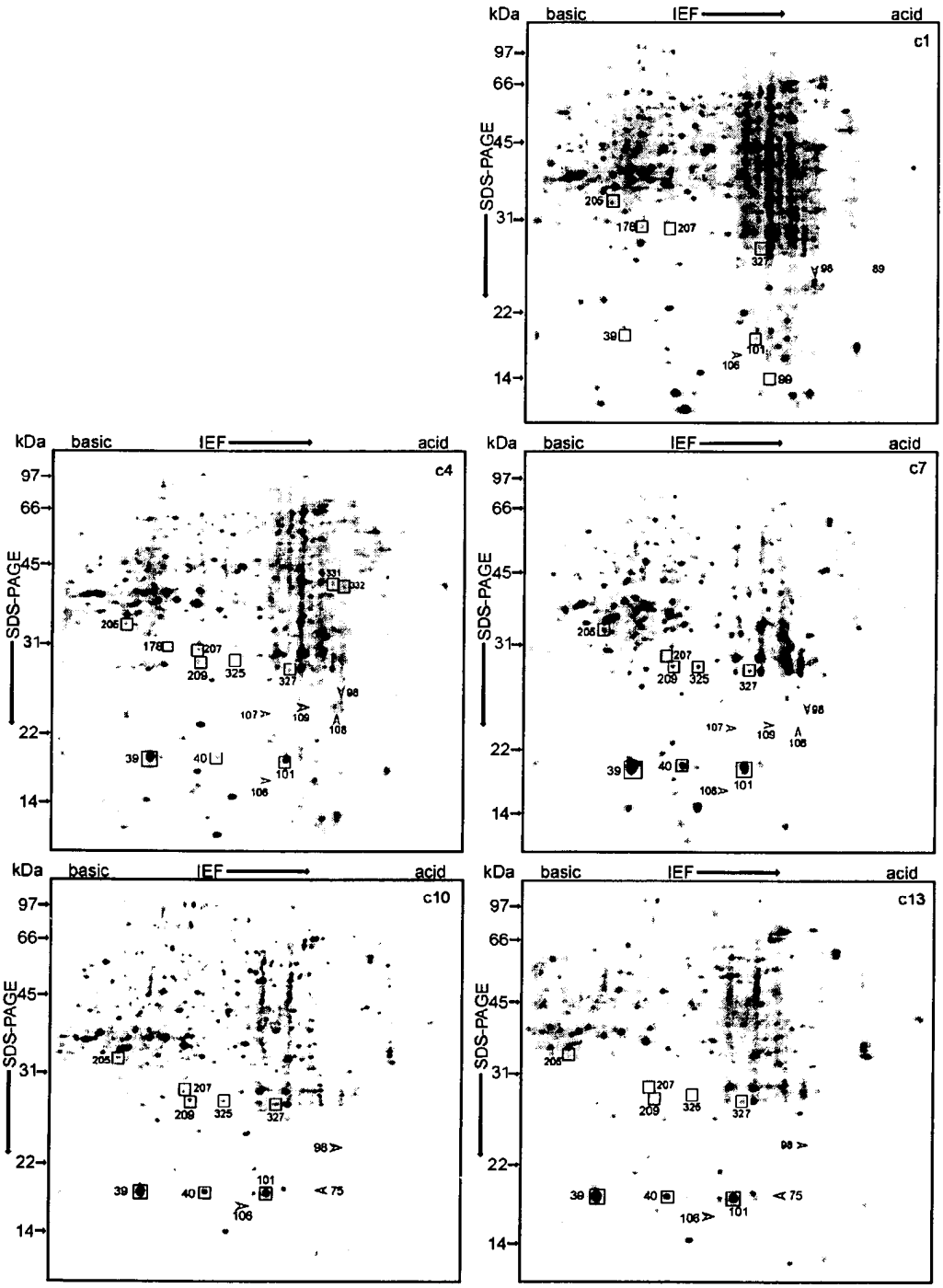

Fig. 4. Silver-stained 2D-PAGE of proteins extracted from rooting region in hypocotyls of radiata pine without IBA (controls) at day 1, 4, 7, 10 and 13. Proteins associated with wounding/tissue culture and newly detected in control only are indicated.

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