Synergistic Interaction Between Coumarin 1,2-benzopyrone and IBA in Stimulating Adventitious Root Formation in *Vigna radiata* (L.) Wilczek Cuttings: II. Peroxidase Activity and Acidic Isoperoxidases and Their Relations to Rooting

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Abstract

Cuttings from 7-day-old *Vigna radiata* seedlings were treated for 24 h with 1000 µM coumarin and/or 50 µM indole-3-butyric acid (IBA), to study their effects on stimulating adventitious root formation (ARF). The effects of treatment on total soluble peroxidase and acidic isoperoxidases (iso-PERs) were also investigated at the potential rooting sites during the first 96 h after application. Simultaneously, combined treatment acted synergistically in inducing more adventitious roots in treated cuttings than in those treated with coumarin or IBA individually, as compared with the control. During the primary events of ARF process, an initial temporary decrease in PER in relation to coumarin or IBA treatments, but an increase in relation to their combination was found. Thereafter, a steady marked increase in PER activity as well as acidic iso-PERs was noted with higher levels in the treated cuttings than in untreated ones, during the secondary events of ARF process. This suggests that acidic iso-PERs may play a crucial role(s) in root initiation and development rather than in root induction. The possible roles of both basic and acidic iso-PERs in their relation to initiation and development during ARF are discussed.

Keywords: acidic isoperoxidases, adventitious root formation, coumarin, indole-3-butyric acid, mung bean cuttings.

Introduction

Adventitious root formation (ARF) takes-place through a series of successive interdependent physiological phases (Jarvis 1986; Berthon *et al.*, 1990; Hartmann *et al.*, 1997) that are associated with changes in endogenous auxin concentrations (Moncousin *et al.*, 1988; Blakesley 1994). Gaspar *et al.*, (1994) suggested that an early and transient increase in endogenous free IAA level occurs during the induction phase, i.e. the time period required for necessary biochemical events preceding the initiation of cell divisions leading to formation of root primordia. They also suggested that the initiation phase of rooting, i.e. continuation of cell division and cell differentiation, is characterised by a reduction in free IAA concentration to a minimum level, whereas growth and differentiation of the new roots is associated with a slight increase in free IAA concentration.

Peroxidases are widely distributed and highly regulated in various tissues and cellular compartments and play important roles in numerous physiological responses including auxin catabolism, oxidation of phenolics to form lignin, cross-linking of hydroxyproline-rich glycoproteins in plant cell walls, catalyse the synthesis of H_2O_2 from NADH and H_2O and catabolism of H_2O_2 and other reactive oxygen species, which may affect ARF at various developmental stages (Mader *et al.*, 1980; Grambow and Langenbeck-Schwich 1983; Alba *et al.*, 1993; Savitsky *et al.*, 1999; Fry 1988; Lagrimini 1991; Legendre *et al.*, 1993; Olson and Varner 1993; Klotz and Lagrimini 1996; and Esteban-Carrasco *et al.*, 2002). Among PERs, acidic isoPERs are considered to play crucial role in ARF process (Gaspar and Hofinger, 1989). Conflicting results have been reported in terms of the role played by acidic PERs with respect to IAA oxidation in vivo, Van der Berg *et al.*, (1983), Ros Barcel *et al.*, 1989, Gaspar *et al.*, 1991, Zheng and Van Huystee 1992) reported that acidic isoperoxidases are involved in IAA oxidation. However, other investigators reported that they participate in the process of tissue differentiation, lignification and xylogenesis (Fukuda and Komamine 1982; Mader and Fussl 1982; Goldberg *et al.*, 1985; Imberty *et al.*, 1985; Miller *et al.*1985; Gaspar *et al.*, 1985). Previous recent investigation carried out by Tartoura *et al.*, (2004) showed that there is a synergistic interaction between coumarin and IBA on stimulating adventitious root production in mung bean cuttings. They added that the interaction between this phenolic compound and IBA in terms of endogenous free and conjugated IAA concentration, basic PER activity

determination and its basic iso-PER analysis during the time-course of ARF were evaluated.

This investigation aimed to study interaction between coumarin and IBA and their effect on inducing ARF. Coumarin and/or IBA treated cuttings were examined for acidic iso-PERs as well as total soluble peroxidases, during the time-course of ARF, with the objective of shedding light on their relation to root initiation and development.

Material and Methods

Plant material and treatments.

This investigation was carried out in a growth chamber of the Faculty of Natural Science, Michigan State University, East Lansing, MI, USA, during 2000 and 2001 seasons. Seeds of mung bean (*Vigna radiata* Wilcz.) obtained from Maijer Company, MI, were soaked in aerated water for 3 h, then germinated in trays and covered lightly with moist vermiculite in darkness for 48 h at 24°C. After germination, the seedlings were placed in a growth chamber 75% relative humidity, with a continuous cool white fluorescent lights at 20 W/m^2 PAR. Five cuttings from 7-day-old uniform seedlings, consisting of terminal bud, one pair of primary leaves, epicotyl and 5 cm of hypocotyls, were placed in 10 ml vials containing a 3 cm depth of distilled water or test solutions of 1000 µM coumarin, 50 µM IBA and their combined application to cuttings for 24 h. These concentrations used in the present study are based on a previous study of Tartoura *et al.*, (2004). The cuttings were subsequently transferred to new vials containing a 5 cm deep of distilled water for 7 days. Distilled water was added daily to each vial to maintain the original solution level. Incubation conditions were identical to those for seedling growth. The time-course appearance of emerging roots through epidermis was recorded and the number of these roots after 7 days was determined. Calculation of the synergistic effect between coumarin and IBA in terms of adventitious root numbers was estimated using the following formula:

where IBA and coumarin represent the number of roots formed during individual treatments, IBA/coumarin that formed during simultaneous treatment and H_2O represent the number of roots formed in the control. Data are mean values ± SD of three independent experiments, each with three replicates consisting of 25 cuttings each.

Sampling, extraction and PER determination

Samples of 1.5 cm length from the basal portions of the cuttings treated with 1000 µM coumarin, 50 µM IBA and their combination were collected at 0, 6, 12, 24, 48, 72, and 96 h during the progress of ARF. Samples were frozen immediately in liquid nitrogen, and stored at -80°C until analysis. The frozen tissues were homogenized in liquid nitrogen at 4°C with a prechilled mortar and pestle in cold 0.2 M K-phosphate buffer, pH 7.0 in the proportion of 1.0 g fresh tissue to 2 ml buffer. The homogenates were centrifuged at 15,000g for 30 min at 4°C and the supernatants were decanted and the pellets were resuspended in the same amount of buffer and centrifuged under the same previous condition. The two supernatants were combined and used to assay enzymatic activity and the separation of acidic PER isozymes by native polyacrylamide gel electrophoresis (PAGE) analysis. Peroxidase activity was assayed according to a modified method of Hammerschmidt *et al.*, (1982). Briefly, the assay mixture in a total volume of 3 ml contained 10 mM K-phosphate buffer, pH 7.5 at 25°C, 2 mM H_2O_2 and 9 mM guiacol as the substrate. After addition of 3 µl of crude enzyme extract, increase in absorbance was measured at 470 nm by a Beckman DU 530 spectrophotometer at intervals of 30s up to 2 min. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard. Activities of PER were expressed as enzyme units min^{-1}mg^{-1} protein.

Non-denaturing PAGE of acidic iso-PERs was performed, by vertical PAGE in an LKB 2001 apparatus, using 7.5% (w/v) polyacrylamide separating gel and 5% stacking gels, respectively. Polyacrylamide gels were buffered with 1.5 M tris-HCl buffer (pH 8.8) according to the method of Keleti and Lederer (1974) with a nondissociating discontinuous buffer system. The gels were run at 4?C at a constant current of 100 V with 0.02% (w/v) bromophenol blue as tracking dyes. After electrophresis, the PAGE gels were equilibrated in 50 mM sodium acetate (pH 5.0) for 20 min followed by incubation at room temperature for 30-60 min in a solution containing 50 mg of 3-amino-9-ethylcarbazole, 10 ml of N,N dimethylformamide, 200 µl of 30% H_2O_2 and 190 ml of 100 mM sodium acetate buffer, pH 5.0 (Grahm *et al.*, 1965). Dark brown bands appeared at the sites of acidic isoPER activity. Gels were rinsed and stored in a solution of 7% acetic acid in 50% methanol. Separation of each sample using PAGE was repeated three times with identical results.

Results

Synergistic interaction of coumarin and IBA

Table 1 shows that adventitious root number increased markedly in response to coumarin and IBA separately when compared to the control. In addition, coumarin acted synergistically with IBA in stimulating more adventitious roots production in treated cuttings than in those treated with coumarin or IBA individually. Table 1 also shows that the time-course appearance of emerging roots through epidermis at the cutting bases was delayed in the following order: control, coumarin, IBA and their combined application. The time of root primordia appearance through the epidermis at the regeneration zones of the cuttings treated with the application of 1000 µM coumarin plus 50 µM IBA,

Table 1. Effect of coumarin and /or IBA on adventitious rooting of *V. radiata* cuttings and time-course appearance of emerging roots through epidermis. The percentage synergy is shown in parenthesis. Data show the means ± SD of three replicates from two independent experiments.

Treatment	Number of adventitious roots/cutting	Time of root emergence through epidermis (h)	Synergy (%)
Water	6.4 (± 0.51)	71.0 (± 3.6)	-
1000 µM Coumarin	31.1 (± 3.25)	77.0 (± 4.0)	-
50 µM IBA	38.7 (± 3.50)	83.0 (± 4.4)	-
1000 µM Coumarin + 50 µM IBA	125.0 (± 9.50)	98.7 (± 3.8)	108.0

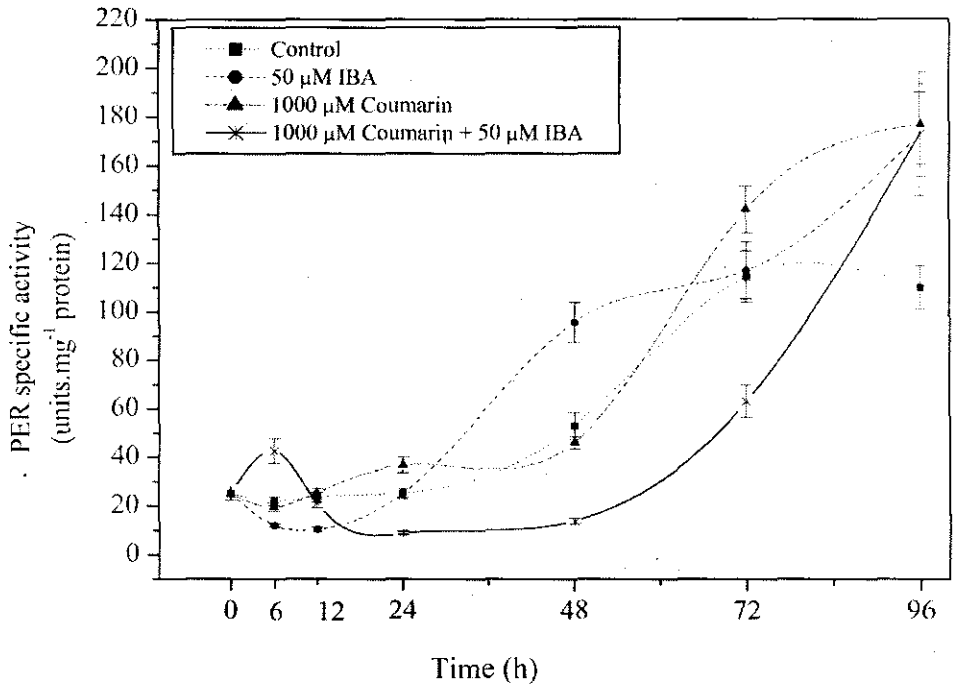

Fig. 1. Changes in specific peroxidase activity levels in the basal portions of *V. radiata* cuttings treated with coumarin and/or IBA during the indicated time-course of ARF. Vertical bars represent ± SD.

was found to be later than those in untreated and other treated cuttings. These results show that the untreated cuttings progressively entered and passed the successive phases of adventitious rooting process that described earlier 27 h earlier than those treated with a combination of coumarin plus IBA in terms of ARF.

Total soluble PER and its acidic isoPERs

An initial slight decrease in total soluble PER activity occurred during 6 h after application of coumarin or IBA separately to cuttings relative to the respective control. However, in response to application of coumarin plus IBA together to the cuttings, an initial slight increase was noted, followed by a decrease with a minimum level in PER activity between 24 and 48 h. Thereafter, a steady marked increase was noted earlier in control cuttings when compared to treated ones, especially those treated with coumarin plus IBA in combination (Fig. 1).

Discussion

Coumarin promotes ARF in many different plant species cuttings (Dhawan and Nanda 1982; Tartoura 1994). The present study shows that the treatment of mung bean hypocotyls cuttings with a combination of coumarin and IBA was more effective in stimulating ARF than when coumarin or IBA was applied alone. These data are similar to that recorded by Pan and Gui (1997), Fletcher *et al.*, (1988), Pan and Zhao (1994), and Tartoura *et al.*(2004). The delayed appearance of adventitious root primordia following treatment with a higher auxin concentration is in agreement with Gronroos

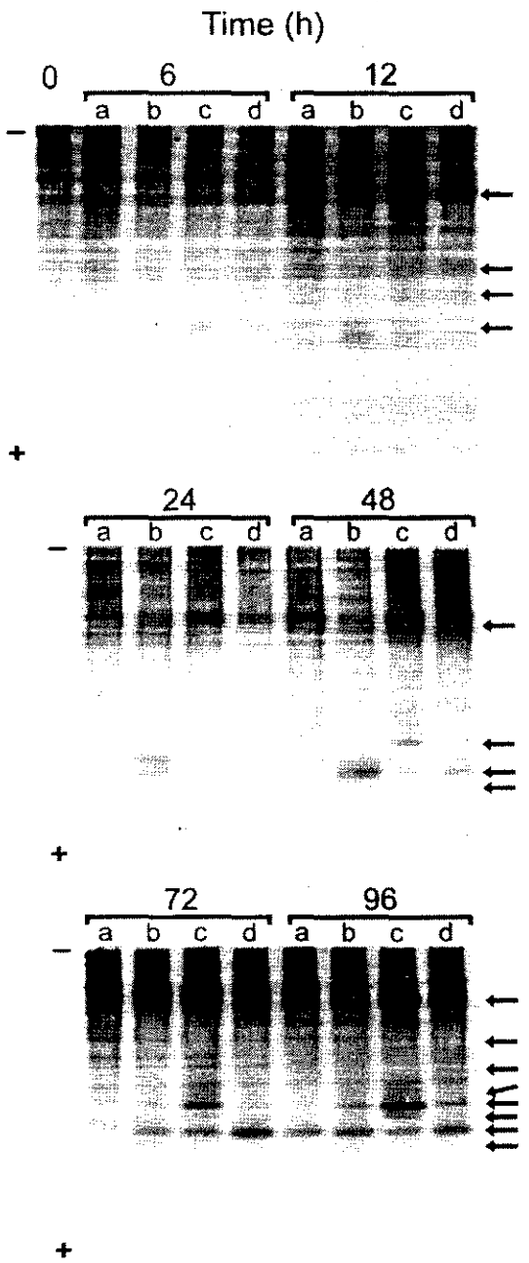

Fig. 2. Acidic isoperoxidase Patterns in the basal portions of *V. radiata* cuttings treated with 1000 µM coumarin, 50µM. and their combined application to cuttings during the indicated time-course of adventitious rooting. Crude enzyme extracts (30µg protein were loaded on 10% PAGE gels.

and Von Arnold (1987) who reported that high auxin concentration might delay root initiation or even inhibit the elongation of adventitious roots. However, the simultaneous combined application used in this investigation was just sufficient to stimulate significantly ARF without causing any side effects.

Peroxidases are found in multiple forms and associated with a diverse of physiological responses including metabolism of auxin and phenolic compounds, cross-linking of cell wall polysaccharides, differentiation of root initial primordia, vascularization, cell wall genesis, ethylene biosynthesis among other processes, which may affect ARF at various developmental stages (Gaspar and Hofinger 1989; Hand 1994, Gaspar *et al.*, 1997). The present results indicate that PER activity had an initial decrease in coumarin or IBA treated cuttings but it showed that an initial increase followed by a decrease in combined application of coumarin plus IBA to cuttings compared to the control, during the primary events of ARF. However, during the secondary events of ARF, the present results suggest that the bulk of total soluble PER activity tended to be more evident and these increases might indicate that PER activity involved in root initiation and development than in root induction as shown in Fig. 1. Similar results were found in beans (Upadhyaya *et al.*, 1986; and Ben-Efraim *et al.*, 1990), hazelnut cotyledons (Gonzales *et al.*, 1991), and V. *radiata* (Tartoura 2001). Indeed, the observed increase in total PER activity during the secondary events of ARF might be attributed to the process of cell wall genesis inherent to cell division and initial root primordia as well as to xylogenesis that takes- place during ARF process, as demonstrated by Wolter and Gorden (1975) and Garcïa-Gömez *et al.*, (1995).

According to Blazich and Heuser (1979) and Jarvis (1986), the adventitious rooting process in mung bean cuttings starts with the induction phase, 0-24h; followed by early phase of root initiation, 24-48h; late initiation phase, 48-72h; and root growth and development, 72 h-onwards). The present investigation do not support the theory proposed by Gaspar and Hofinger (1989) who suggested that adventitious root initiation occurred at the potential sites of root formation after a peak of maximum PER activity, during the induction phase that is characterized by a lack of cell division. In agreement with the present results, Jarvis (1986) placed the root induction phase before increasing PER activity during the secondary events of ARF. Further studies by many investigators confirm the latter view (Moncousin *et al.*, 1988; Pythoud and Buchala 1989; Gaspar *et al.*1994). The latter authors explained the disappearance of PER activity during the root induction phase in some studies to: either PER activity units were not expressed as units mg $^{-1}$ protein or PER activity units were determined in highly purified extracts. In the present study, PER activity peak during the induction phase of rooting is not present even when PER activity was determined in a non purified extract and/or PER activity was expressed as units mg $^{-1}$ protein. However, an initial increase in the cuttings given a simultaneous application of coumarin plus IBA was noted (Fig. 1) and that may be attributed to catabolism of the supraoptimal amounts of the IBA

and coumarin that were applied to the cuttings to induce ARF. One may conclude that the drop in temporarily often accumulated IAA during the root induction phase is due to increasing IAA-O/PER activities, but our findings in the present investigation and a previous study (Tartoura *et al.*, 2003) do not agree with this suggestion. In fact, the comparison of the dynamics of accumulation of conjugated forms of IAA and activity of basic iso-PERs, that thought to be responsible for IAA oxidation in vivo, as well as acidic iso-PER, see later, leads to conclude that the former but not the latter is responsible for down-regulation of endogenous IAA levels that accumulated during the induction phase of rooting.

The PER minimum level that was found in cuttings treated with coumarin or IBA individually, during the early phases of adventitious rooting (Fig. 1), was also previously noted in other works (Moncousin *et al.*, 1988; Ripetti *et al.*, 1994; Gaspar *et al.*, 1994). Those authors attributed the transient increase in IAA levels during the induction phase of rooting to lowering IAA-O/PER activities. Interestingly, this minimum level has been observed following exogenous application of the cytokinin benzylaminopurine (N^6 BAP) to mung bean cuttings. N^6 BAP inhibits significantly adventitious rooting in mung bean cuttings (results not shown). This indicates that there is no any relation between accumulation of IAA during the early phases of adventitious rooting and the initial decrease in PER activity or vice versa during the early initiation phase of ARF. In fact, the principle oxidation pathway in V. *radiata* does not appear to involve PERs (Gus'Kove *et al.*, (1980). Those workers found that free IAA was rapidly converted to auxin conjugates. The same findings was also found by Plüss *et al.*, (1989), Nordstrom and Eliasson (1991), and Garcïa-Gömez *et al.*, (1994). Thus, the relation between initial lowering PER activity and ARF has to be reexamined, as suggested by Gaspar *et al.*, (1997).

Considering the role of PERs in regulation of endogenous free and conjugated IAA, it has been found that conjugated IAA level started to decrease immediately after reaching a maximum level, but free IAA level did not significantly change (Garcïa-Gömez *et al.*, 1994; Tartoura *et al.*, 2001). This may be explained by the fact that some conjugated IAA might be hydrolysed at the later stages of the rooting process and the released IAA is rapidly oxidized by PER activity when high auxin activity is required for optimal rooting, as suggested by Nordstrom *et al.*, (1991) and/or conjugated IAA might be subjected to a direct PER-mediated IAA oxidation during the progress of ARF. Indeed, basic PER and its basic isoforms (Tartoura *et al.*, 2004) or PER activity and its acidic isoforms in this investigation (Fig. 1 and 2) that are maintained at high levels of activity during the later stages of adventitious root production, might be involved in free IAA oxidation as reported by Gazaryan *et al.*, (1996) or in conjugated IAA oxidation, as also suggested by Plüss *et al.*, (1989), Tsurumi and Wada (1990), and Tuominen *et al.*, (1994). Thus, it may be concluded that basic and acidic iso-PERs are involved in free and conjugated IAA oxidation in vivo during the secondary event of ARF, as also reported by Van der Berg *et al.*, (1983), Zheng and Van Huystee (1992), and Garcïa-Gömez *et al.*, (1995). Indeed, these isoperoxidases could link auxins and the mechanisms that lead to cell wall rigidity by means of auxin and phenolic oxidation and lignification. These biosynthetic processes would be required during formation of the new xylem elements and the epidermal surfaces of the newly formed organs, as suggested by van der Berg *et al.*, (1983), Imberty *et al.*, (1985), Hagege *et al.*, (1988), and Mohan and Kolattukudy (1990), Zheng and Van Huystee (1992) and Mc Dougall (1992). However, it may be suggested that iso-PERs are also involved in numerous biochemical reactions other than those associated with lignification, as previously stated.

Electrophoretic patterns of acidic isoPERs are well related with total PER activity that was previously discussed (Fig. 1 and 2). That means that increases or decreases in acidic PER activity is accompanied with increases or decreases in the intensity and number of enzymatic bands, especially among the various stages of adventitious rooting process. For example, early initiation phase occurring during the second 24 h after treatment, as can be inferred from the histological studies conducted by Chandra *et al.*, (1971) and Blazich and Heuser (1979), was associated chronologically with a decrease in acidic iso-PER activity. However, the activity of acidic iso-PERs was increased later during late initiation, growth and development of adventitious roots. Acidic iso-PER bands shown in Fig. 2 do not show an increase in the intensity or number of acidic iso-PER bands during the secondary events of ARF even with the simultaneous application between coumarin and IBA that acted synergistically in initiating more adventitious roots production (Table 1). Extending the time-course of analysis may reveal new unique bands since emergence of the new roots were delayed in the combined treatment about 27 h compared to untreated or other treated cuttings. Indeed, 120 h analysis revealed 3 more bands in the combined treatment against one in the IBA treatment (data not shown).

Conclusion

It could be concluded that coumarin acted synergistically with IBA in stimulating more adventitious roots production in mung bean *V. radiata* cuttings. In addition, there was a positive relation between the level of PER activity and its acidic iso-PER during the secondary events of ARF and the number of adventitious roots formed. Further, acidic iso-PERs together with basic iso-PERs did not appear to participate in a

principle way in the degradation of endogenous free IAA that elevates transiently during the early phases of adventitious rooting process but both may be involved in the oxidation of free and conjugated IAA during the secondary events of ARF. Thus, the observed increase in PER activity may play a role in auxin catabolism. The relatively high levels of conjugated IAA during the primary events of ARF also suggest that the formation of conjugated IAA is the main mechanism participated in auxin catabolism in mung bean cuttings.

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التأثير التنشيطى التعاوني بين الكيومارين ٢، ١ بنزوبيرون و إندول حمض البيوتيرك فى تكوين الجذور لعقل اللوبيا: ـ. علاقة إنزيمات البيروكسيديز و مشابهاته الحمضية بتكوين الجذور العرضية

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أجريت هذه الدراسة بهدف تفسير التأثير التنشيطي التعاوني بين الكيومارين و اندول-٣-حمض البيوتيرك علي تكوين الجذور العرضية لعقل اللوبيا و أيضا علي محتوي قواعد تلك العقل من انزيم البيروكسيديز و مشابهاته الحمضية خلال مراحل تطورية مختلفة لتكوين تلك الجذور. أظهرت النتائج المتحصل عليها أن مخلوط الكيومارين و اندول-٣-حمض البيوتيرك بتركيزات مبنية علي دراسة سابقة وهي ١٠٠٠ ميكرومولار بالنسبة للكيومارين، ٥٠ ميكرومولار بالنسبة لإندول-٣-حمض البيوتيرك و لمدة ٢٤ ساعة أدت إلي تحفيز عدد أكثر من الجذور المتكونة علي العقل بمقارنتها بلكيومارين أو اندول-٣-حمض البيوتيرك كلا علي حدة. بالإضافة إلي ذلك أوضحت النتائج أيضا أن هناك زيادة مستمرة بدرجة ملحوظة لنشاط البيروكسيديز و مشابهاته الحمضية في العقل المعاملة مقارنة بغير المعاملة أثناء المراحل المتأخرة لتكوين الجذور مما أدي غلي استنتاج أن مشابهات البيروكسيديز الحمضية تلعب أدوارا هامة في تكوين و تطور الجذور العرضية بالمقارنة بدورها فى إحداث induction لتلك الجذور. تم مناقشة دور مشابهات البيروكسيديز القاعدية و الحمضية في تكوين الجذور العرضية