Rev. Biol. Trop. 49(3-4): 965-971, 2001
www.ucr.ac.cr www.ots.ac.cr www.ots.duke.edu

Onset of *in vitro* rhizogenesis response and peroxidase activity in *Zingiber officinale* (Zingiberaceae)

G.R. Rout, S.K. Palai and P. Das
Plant Biotechnology Division, Plant Tissue Culture Laboratory, Regional Plant Resource Centre, Bhubaneswar- 751 015, India. Fax: 0091-674-550274. E-mail: grrout@hotmail.com

Received 07-X-2000. Corrected 10-V-2001. Accepted 28-V-2001.

Abstract: The induction of rooting in microshoots of *Zingiber officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18} was achieved on half-strength basal Murashige and Skoog's medium supplemented with 0.5-1.0 mg/l either indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) and 2 % (w/v) sucrose within 7-9 days of culture. Rooting was inhibited when the microshoots were cultured under higher concentration of auxins. The microshoots cultured on medium supplemented with NAA induced large number of thin root hairs with friable calluses within 6-7 days. Peroxidase activity was determined during root induction (0-day to the 10^{th} day at every 2 day interval) from microshoots derived *in vitro*. The activity was minimum in the inductive phase (primary) and at the maximum level during the root initiative phase. These finding may be useful in monitoring the rooting behaviour in microshoots derived from different subculture and peroxidase activity as a marker for root initiation.

Key words: *In Vitro*, medicinal plant, peroxidase activity, root initiation, shoot multiplication.

Ginger, *Zingiber officinale* Rosc. (Zingiberaceae), is an important, tropical, horticultural plant valued in culinary and pharmaceutical preparations. Cultivation of the plant is limited by diseases spread through vegetative propagation and limiting supply of high quality rhizomes. The quality of the rhizome depends on the age and a the harvesting time. The oil content in plantlets derived from *in vitro* cultures could also be controlled by the composition of the culture medium. High-quality volatile oils and higher rhizomes yield in ginger would be of great benefit to pharmaceutical industries. *In vitro* culture techniques provide an alternative way of plant propagation and function as a tool for crop improvement programs (Vasil 1988). Rooting of microshoots is critical in plant production systems *in vitro*. Induction of rooting depends on a series of interdependent phases (induction, initiation and expression) (Gaspar *et al.*, 1994). Various studies on adventitious root formation have shown the fundamental role played by peroxidase in rooting of plants cultured *in vitro* (Berthon *et al.* 1989, Rival *et al.* 1997). The effect of auxins with regard to the peroxidase activity in rooting of various plant species has also been reported by Kevers *et al.* (1997). The aim of the present investigation is to describe the effect of auxins on rooting and analyse the peroxidase activity during root induction in microshoots of *Z. officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18}.

MATERIALS AND METHODS

Plant material: Mature rhizomes of *Z. officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18} were collected at the High Altitude Research Station of the Orissa University of Agriculture and Technology, Pattangi, Orissa, India and incubated in a bed of sterile, moist, sandy soil (65 % relative humidity with

26±2 ºC) to induce sprouting. The pale-white sprouting buds (after 30 days of incubation) were collected and washed with 2 % (v/v) detergent solution 'Teepol' (Qualigen, India) for 15 min, subsequently surface sterilised using 0.1 % (w/v) aqueous solution of mercuric chloride for 25 min followed by several rinses in sterile distilled water. The shoot buds were dissected under a dissecting microscope; the meristems with apical dome or with one or two leaf primordia ranging in size from 0.1-0.5 mm were removed and used as explants.

Culture medium: Isolated shoot meristems were placed on Murashige and Skoog (1962) (MS) basal salts supplemented with various concentrations of cytokinins, *i.e*. 6-benzylaminopurine (BA: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 mg/l), and adenine sulfate (Ads: 50, 100, 150 and 200 mg/l), and auxins like indole-3-acetic acid (IAA: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), indole-3-butyric acid (IBA: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) and 1-naphthaleneacetic acid (NAA: 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) for shoot multiplication. The pH of the medium was adjusted to 5.8 prior to autoclaving. All cultures were incubated under a 16 hr photoperiod having a light intensity of 55 µmol $m^{-2}s^{-1}$ from cool, white fluorescent lamps at 25 ± 2 ºC.

Induction of rooting and transplantation: Microshoots (1-2 cm) were separated from the initial culture as well as different subcultures and transferred into various semi-solid basal half-strength MS medium alone or in combination with IAA, IBA or NAA (0.0, 0.25, 0.50, 1.0 and 1.5 mg/l) and 2 % (w/v) sucrose for root induction. In another set of experiments, the microshoots derived from the multiplication medium were cultured for 7 days on basal MS medium and subsequently transferred to the rooting medium (1/2 MS + 0.5-1.0 mg/l IAA or IBA + 2 % (w/v) sucrose) for induction of rooting. The incubation conditions were the same as mentioned earlier for shoot growth. The rooted plantlets were removed from the culture vessels, washed gently under running tap water and planted in earthen pots containing a mixture of soil and sand (3: 1) (v/v). The plantlets were acclimatized for one month in the greenhouse (85 % relative humidity at 30 ± 2 ºC).

Sample collection: Microshoots were collected from the mother cultures prior to inoculation into the rooting medium (0-day) and at every two day intervals up to 10 days. The experiment was repeated three times.

Enzyme extraction and assay: Peroxidase activity: For determination of peroxidase activity, samples (rooting zone of the microshoots) (100 mg) were collected at 2-day intervals and homogenised in chilled mortar and pestle with 4 ml cold 0.1 M phosphate buffer (pH 6.1) containing 30 mg of insoluble PVP (polyvinylpyrrolidone) and 15 mg sodium ascorbate. The homogenate was filtered through four layers of miracloth and centrifuged at 12 000 $\bar{g}$ and 4 ºC for 10 min; the supernatant was used for the peroxidase assay. The assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol as donor, 3 mM H_2O_2 as substrate and 0.4ml crude enzyme extract. The total reaction volume was 1.2 ml. The rate of change in absorbency (OD) at 420 nm was measured using a UV-Spectrophotometer (UVIDEC-650, Jasco, Japan). The levels of enzyme activity were expressed as mmol (H_2O_2 destroyed) mg^{-1} (protein) s^{-1} (Bergmeyer *et al.* 1974). Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Scoring of data and statistical analysis: All the cultures were examined periodically. The initiation of root from microshoots was recorded on the basis of visual observations. There were 20 replicates per each treatment and the experiment was repeated at least three times. Subculturing of shoot multiplication was carried out at an interval of four weeks. The effects of different treatments were quantified on the basis of percentage of culture showing response for multiplication, rooting and the number of roots/shoot. The data were analysed statistically by the Duncan's Multiple Range test (Harter 1960).

RESULTS

Shoot multiplication: Multiplication of shoot was achieved from apical meristems of *Z. officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18} on MS basal medium containing 4.0-6.0 mg/l BA supplemented with 1.0-1.5 mg/l IAA and 150 mg/l adenine sulfate. The rate of shoot multiplication of different cultures reached a maximum value on medium having 6.0 mg/l BA, 1.5 mg/l IAA and 150 mg/l adenine sulfate (Fig. 1a). Of the three auxins tested, IAA was the most effective for shoot proliferation (data not shown). There was no sign of shoot multiplication when explants were cultured in media devoid of cytokinin. At lower concentrations of BA, the rate of shoot proliferation declined. The rate of shoot multiplication was stable and constant up to one year without declination.

Induction of rooting from microshoots: Elongated microshoots (1-2 cm) were rooted on half-strength basal MS medium supplemented with various concentrations of either IAA or IBA depending on the cultures tested (Table 1). The rooting in the microshoots was inhibited in the medium devoid of growth regulator. Though NAA induced roots at lower concentration, callusing appeared at the basal cut end. Root initials were observed within 7-8 days of culture which developed into a good root system within 8-10 days of culture (Fig. 1b). Rooting ability was slowed on the medium containing higher concentrations of IAA or IBA (1.0-1.5 mg/l). However, optimal rooting in 'Suprava', 'Turia local', 'Suruchi' and

TABLE 1

Effect of different concentrations of IAA and IBA on root induction from microshoots of Zingiber officinale *cvs. Suprava, Turia local, Suruchi and* V_3S_{18}

Zingiber officinale cvs		MS + 2 % Sucrose + different concentrations of IAA and IBA								
			IAA (mg/l)				IBA(mg/l)			
		0	0.25	0.50	1.0	1.5	0.25	0.50	1.0	1.5
Suprava	A	0	46.0	68.4	87.2	58.3	32.3	44.2	57.8	46.4
			±2.3	±1.5	±1.4	±2.3	±1.6	±1.7	±1.4	±0.8
	B	0	5.36	6.42	7.32	6.2	4.21	4.84	5.21	5.32
	C	0	10-11	8-9	7-8	8-9	11-12	10-11	9-10	10-11 c
Turia local	A	0	44.4	82.4	90.6	60.6	32.6	44.8	67.4	52.6
			±1.6	±1.5	±1.7	±1.4	±1.6	±1.4	±1.3	±1.5
	B	0	6.56	8.36	8.42	4.23	3.11	3.82	6.12	4.52
	C	0	11-12	8-9	7-8	12-13 c	13	11-12	12	10-11 c
Suruchi	A	0	32.0	64.2	96.4	72.4	24.3	40.0	64.4	42.8
			±1.4	±1.3	±1.5	±1.4	±1.6	±1.2	±1.1	±1.4
	B	0	4.24	6.56	9.21	5.23	3.21	4.36	5.24	3.38
	C	0	12-13	10-11	7-8	11-12 c	12	10-11	11	9-10 c
V_3S_{18}	A	0	66.7	74.4	70.4	60.2	68.6	84.2	76.8	64.3
			±1.5	±1.8	±1.4	±0.9	±0.7	±1.4	±0.8	±1.7
	B	0	6.72	7.36	6.42	4.36	6.82	8.36	4.34	3.86
	C	0	10	8-9	8-9	10-11 c	7-8	6-7	7-8	10-11 c

A- Percentage of shoots rooted (Mean ± S.E.)*; B- No. of roots/shoot; C- Days to rooting.

* 20 replicates per treatment; repeated three times.

c – callusing at the basal cut end.

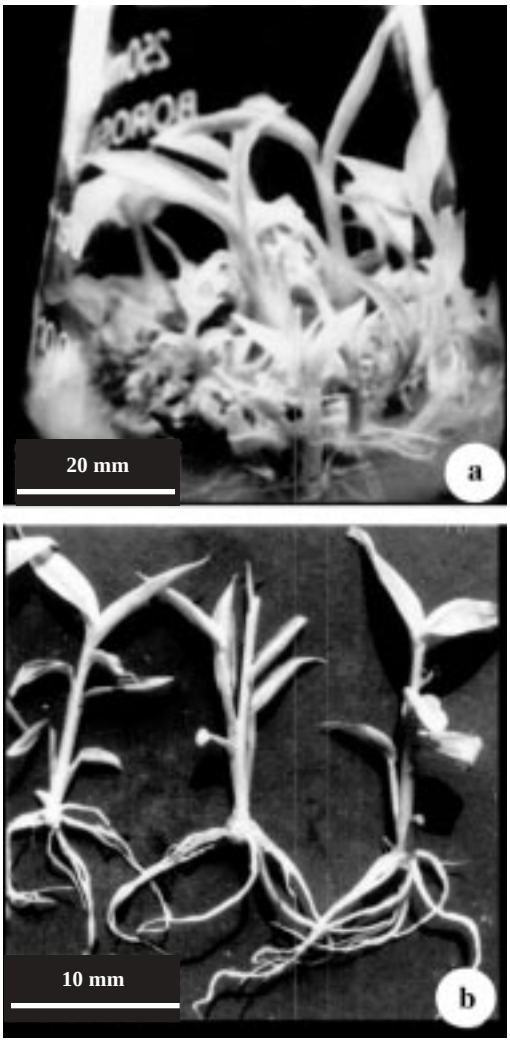

Fig. 1. a. Induction of multiple shoots from shoot meristems of *Zingiber officinale* cv. Turia local on MS medium supplemented with 6.0 mg/l BA, 1.5 mg/l IAA and 150 mg/l adenine sulfate after 4 weeks of culture (Bar = 20 mm). b. Induction of rooting from microshoots of *Zingiber officinale* cv. Turia local on $^1/_2$ strength MS semisolid medium supplemented with 1.0 mg/l IAA after 10 days of culture (Bar = 10 mm).

'V_3S_{18}' was 87.2, 90.6, 96.4 and 70.4 respectively in medium containing 1.0 mg/l IAA. The percentage of microshoots forming roots and the number of roots/shoot varied significantly with different concentrations of IAA or IBA; the shoots developed root hairs with intervening calluses at higher concentrations. The rooting ability was decreased when microshoots derived from the 4th subculture. The microshoots grown on a cytokinin rich medium and subsequently transferred to rooting medium did not give any significant response for induction of rooting. The microshoots grown on a hormone free medium for 7 days and subsequently transferred to rooting medium showed positive response.

Peroxidase activity: The peroxidase activity in microshoots subjected to different treatments during the rooting process was determined (Table 2). The activity was at the minimum in the primary (inductive) phase and at the maximum in the secondary (initiative) phase in the microshoots grown on medium having 0.25-1.0 mg/l IAA or IBA (Table 2). The minimum activity was noted between the 0 and 2nd day; the maximum activity, however, was noted from the 6th to the 8th day of culture.

Acclimatization and field establishment: About 95 % of the rooted plantlets established in the greenhouse within two-three weeks of transfer. The plants grew normally and attained six-eigth cm within 30 days of transfer.

DISCUSSION

The present study showed that it is possible to induce multiple shoots and rooting from apical meristems of *Z. officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18} on medium containing different concentrations of cytokinin and auxin. The combination of auxin and cytokinin influencing the production of shoots and the subsequent elongation of some ginger cultures was well documented (Malamug *et al.* 1991). The multiple shoots were rooted on half-strength MS semisolid medium supplemented with 0.5-1.0 mg/l IAA or IBA within 7-8 days of culture. The percentage of microshoots forming roots and the number of roots/shoot varied significantly with different concentrations of IBA or IAA; the

TABLE 2

Peroxidase activity in microshoots of Zingiber officinale *cvs. Suprava, Turia local, Suruchi and* V_3S_{18} *in the absence and presence of different concentrations of IAA and IBA prior to inoculation on rooting media (0-day) and after inoculation on rooting media at 2-d, 4-d, 6-d, 8-d and 10-d of culture*

Zingiber officinale cvs.	Culture period (days)	Peroxidase activity (mmoles H_2O_2 destroyed/min/mg protein)								
		Concentration (mg/l)								
			IAA				IBA			
		0	0.25	0.5	1.0	1.5	0.25	0.5	1.0	1.5
Suprava	0	4.34	4.43	5.23	5.12	5.65	3.42	3.51	3.82	3.71
	2	5.12	5.17	6.51	7.56	7.24	4.52	4.92	6.11	5.90
	4	5.82	6.42	8.17	8.41	8.27	5.44	5.56	7.85	6.10
	6	6.26	7.27	9.22	10.42	9.34	6.12	6.34	8.13	6.58
	8	6.77	8.16	9.77	12.12	9.41	7.20	7.84	8.61	6.82
	10	7.11	6.45	7.22	9.21	7.31	6.52	6.32	8.22	5.80
Turia local	0	3.32	3.50	4.33	4.02	5.05	3.42	3.61	3.72	4.01
	2	4.12	4.26	6.21	6.56	6.44	4.22	4.85	5.97	5.10
	4	4.80	5.42	7.37	7.46	7.18	6.04	7.20	6.95	5.90
	6	5.24	6.38	7.96	8.52	8.24	7.41	8.74	7.26	6.48
	8	5.70	7.22	8.77	9.62	8.51	8.42	9.80	8.32	6.62
	10	6.13	5.62	6.22	8.31	7.21	5.12	6.12	7.36	5.82
Suruchi	0	3.34	3.53	3.82	4.52	4.25	3.22	3.61	3.82	3.91
	2	4.82	5.27	6.00	6.86	6.24	4.42	4.02	5.11	4.90
	4	5.62	6.40	7.27	7.41	8.02	6.04	5.26	7.25	6.40
	6	6.16	7.12	8.02	9.40	9.34	7.12	7.32	8.43	7.38
	8	8.72	9.06	8.67	10.32	9.82	8.40	9.80	9.61	8.82
	10	7.26	6.60	7.32	9.11	7.20	6.12	7.34	7.32	5.80
V_3S_{18}	0	4.24	4.46	4.80	5.13	5.45	3.22	3.41	3.62	3.61
	2	5.32	4.87	6.12	6.56	6.74	4.42	4.82	5.21	5.83
	4	6.80	5.42	7.13	7.51	8.07	5.34	5.26	6.85	6.93
	6	7.28	7.37	8.22	9.22	9.14	7.12	7.38	8.23	7.68
	8	9.68	8.26	9.67	10.62	10.44	8.40	9.80	9.61	8.72
	10	6.21	5.55	7.12	8.31	7.32	6.42	6.20	7.62	6.20

15 replicates/treatment; repeated thrice.

shoots developed roots with intervening calluses at higher concentrations. The application of exogenous auxins promoting rooting in different plant species was reported (Blazkova *et al.* 1997, Kevers *et al.* 1997). The rooting ability decreased when microshoots derived from the 4th subculture. There are few reports on the effect of age of microshoots on rooting behavior (DeKlerk *et al.* 1997). Enzymes are known as metabolic markers which change during development and differentiation (Scandalios 1969). It is clear that auxin treatments induced a sharp increase in the peroxidase activity during the course of the experiment and this might be due to absorption of auxin during the multiplication stage. Some researchers suggest that auxin enters the cut surfaces and it is rapidly taken up in the cell wall by pH trapping (Rubery and Sheldrake 1973) and by influx carriers (Delbarre *et al.* 1996). Epstein and Ludwig-Muller (1993) reported that during the course of root induction, there are two major pathways of conversion: oxidation and conjugation. Exogenous auxins applied to microcuttings became oxidised, resembling the enzymes involved in the wounding reactions as reported by DeKlerk *et al.* (1999). They also indicated that IAA or IBA oxidation caused by non-specific

peroxidases is related to the wounding response. The peroxidase activity was also at a minimum in the primary (inductive) phase and at the maximum level in the secondary (initiative) phase in the microshoots grown on medium having 0.5-1.0 mg/l IAA or IBA. The minimum activity was noted between day 0 and 2; the maximum activity, however, was noted from the 6^{th}-8^{th} day. The pattern of enzyme activity was similar as reported earlier in other plant systems (Berthon *et al*. 1990, Rival *et al*. 1997). Changes in the levels of peroxidase activity in relation to auxin treatment have been reported in other plants (Moncousin *et al*. 1988, Gaspar *et al*. 1994).

The present study has confirmed the role of auxins on induction of rooting ability of *Z. officinale* cvs. Suprava, Turia local, Suruchi and V_3S_{18} and showed the increased level of peroxidase activity at different intervals during the process of rhizogenesis. Various studies on other plant species (*Asparagus*, *Populus* and *Prunus*) have shown the fundamental role played by peroxidase activity during root initiation and expression (Hausman 1993). The IAA or IBA oxidation is caused by peroxidases related to the wounding response as reported by DeKlerk *et al.* (1999). This study may be useful in monitoring the rooting beahaviour in microshoots and to characterize the molecular events during rhizogenesis.

ACKNOWLEDGMENTS

The authors wish to acknowledge to the Department of Forest and Environment, Government of Orissa for necessary facilities.

RESUMEN

La inducción de enraizamiento en microtallos de *Zingiber officinale* cvs. Suprava, Turia local, Suruchi y $V_3 S_{18}$ fue lograda en medio de Murashige y Skoog basal de dureza media suplementado con 0.5-1.0 mg/l ya sea con ácido 3-indol acético (IAA) o ácido 3-indol butírico (IBA) y 2 % (w/v) de sucrosa dentro de 7-9 días de cultivo. El enraizamiento fue inhibido cuando los microtallos fueron cultivados bajo concentraciones más altas de auxinas. Los microtallos cultivados en un medio suplementado con NAA indujeron un gran número de pelos de raíz delgados con callos frágiles dentro de 6-7 días. La actividad de la peroxidasa fue determinada durante la inducción de enraizamiento (de 0 a 10 días, cada 2 días) de microtallos derivados *in vitro*. La actividad fue mínima en la fase inductiva (primaria) y máxima en la fase inicial de raíz. Estos resultados pueden ser útiles para monitorear el comportamiento de enraizamiento en microtallos derivados de diferentes subcultivos y la actividad de la peroxidasa como un marcador de inicio de enraizamiento.

REFERENCES

Berthon, J.Y., R. Maldiney, B. Sotta, T. Gaspar & N. Boyer 1989. Endogenous levels of plant hormones during the course of adventitious rooting in cuttings of *Sequoiadendron giganteum in vitro*. Biochem. Physiol. Pflan., 184: 405-412.

Bergmeyer, H.U., K. Gaweh & M. Grassl. 1974. Enzymes as biochemical reagents, p. 425-522. *In* H.U. Bergmeyer (ed.). Methods in enzyme analysis. Academic, New York.

Blazkova, A., B. Sotta, H. Tranvan, R. Maldiney, M. Bonnet, J. Einhorn, L. Kerhoas & E. Miginiac. 1997. Auxin metabolism and rooting in young and mature clones of *Sequoia sempervirens*. Physiol. Plant. 99: 73-80.

Bradford, M.M . 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

DeKlerk, G.J., J. Ter Brugge & S. Marinova. 1997. Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in *Malus* 'Jork 9'. Plant Cell Tiss. Org. Cult., 49: 39-44.

DeKlerk, G., W.V.D. Krieken & J.C. DeJong. 1999. The formation of adventitious roots: New concepts, new possibilities. *In Vitro* Cell Dev. Biol. Plant 35: 189-199.

Delbarre, A., P. Muller, V. Imhoff & J. Guern. 1996. Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension-cultured tobacco cells. Planta 198: 532-541.

Epstein, E. & J. Ludwig-Muller. 1993. Indole-3-butyric acid in plants occurrence, synthesis, metabolism and transport. Plant Physiol. 88: 382-389.

Gaspar, T., C. Kevers, J.F. Hausman, J.Y. Berthon & V. Ripetti. 1992. Pratical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. Agronomie 12: 757-765.

Gaspar, T., C. Kevers, J.F. Hausman, J.Y. Berthon & V. Ripetti. 1994. Peroxidase activity and endogenous free auxin during adventitious root formation, p. 289-298. *In* P.J. Lumsden, J.R. Nicholas & W.J. Davies (eds.). Physiology, growth and development of plants in culture. Kluwer Academic, Dordrecht.

Harter, H.L. 1960. Critical values for Duncan's Multiple Range test. Biometric 16: 671-685.

Hausman, J.F. 1993. Changes in peroxidase activity, auxin level and ethylene production during root formation by poplar shoots raised *in vitro*. Plant Growth Regul. 13: 263-268.

Kevers, C., J.F. Hausman, O. Faivre-Rampant, D. Evers & T. Gaspar. 1997. Hormonal control of adventitious rooting: Progress and questions. Angew. Bot. 71: 71-79.

Malamug, J.J.F., H. Inden & T. Asahira. 1991. Plantlet regeneration and propagation from singer callus. Sci. Hortic. 48: 89-97.

Moncousin, C., J.M. Favre & T. Gaspar. 1988. Changes in peroxidase activity and endogenous IAA levels during adventitious root formation in vine cuttings, p. 331-337. *In* M. Kutacek, R.S. Bandurski & J. Krekule (eds.). Physiology and biochemistry of auxins in plants. Academia, Praha.

Murashige, T. & F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473-497.

Rival, A., F. Bernard & Y. Mathieu. 1997. Changes in peroxidase activity during *in vitro* rooting of oil palm (*Elaeis guineensis* Jacq.). Sci. Hort. 71: 103-112.

Rubery, P.H. & A.R. Sheldrake. 1973. Effect of pH and surface change on cell uptake of auxin. Natur. New Biol. 244: 285-288.

Scandalios, J.W. 1969. Isozyme in development and differentiation. Ann. Rev. Plant Physiol. 25: 255-258.

Vasil, I.K. 1988. Progress in the regeneration and genetic manipulation in crops. Biotechnology 6: 397-402.