ROLE OF CELL WALL STIFFENING ENZYMES IN INTERNODE DEVELOPMENT OF *MERREMIA EMARGINATA*

D. Patel and V. Thaker*

Department of Biosciences, Saurashtra University, Rajkot 360 005, India

Received 3 August 2006

Summary. *Merremia emarginata* plants were treated with different plant growth regulators (PGRs): naphthyl acetic acid (NAA), phenyl acetic acid (PAA) and gibberellic acid (GA_3). To determine the role of wall stiffening enzymes in internode elongation, IAA oxidase and peroxidase in the presence of different hydrogen donors like caffeic acid, chlorogenic acid and guaiacol were assayed. The levels of total, mono- and dihydroxy phenols were also determined together with the assay for the presence of IAA protectors. There was no significant difference in internode length upon PGRs treatment. Peroxidase assayed using caffeic acid as a hydrogen donor after GA_3 treatment showed inhibition in both cytoplasmic and wall-bound fraction. Promotion in the activities of the cytoplasmic and wall-bound fractions was observed in NAA and PAA-treated internodes with the other two substrates. IAA oxidase activity was significantly inhibited by GA_3 treatment in the wall-bound fractions.The IAA protectors correlated well with length in control internodes. Total phenols and diphenols showed almost similar contents in control and treated internodes while monophenols were remarkably inhibited by hormone treatments. The possible role of these parameters in elongation growth of the internodes is discussed.

*Corresponding author, e-mail: vrindathaker@yahoo.co.in

Key words**:** IAA oxidase, IAA protectors, peroxidase, phenols, *Merremia emarginata,* wall stiffening.

Abbreviations: Gibberellic acid - GA_3, indole acetic acid – IAA; naphthyl acetic acid - NAA; phenyl acetic acid – PAA; plant growth regulators - PGRs.

INTRODUCTION

Peroxidases are heme-containing glycoprotein enzymes ubiquitous in the plant kingdom. These enzymes catalyze oxidative cross-linking of phenolic groups in the cell wall, and such action might make the wall less extensible (Fry 1986; Schopfer, 1996). Peroxidase may also catalyze the insolubilization and possible crosslinking of wall structural proteins, such as hydroxyproline-rich glycoproteins (Showalter, 1993; Otte and Barz, 1996).

Peroxidase can use various substrates, which act as hydrogen donors *in vitro*, in the presence of H_2O_2. Peroxidase can act as IAA oxidase to control the endogenous level of auxin in the system and thus, it is involved in growth regulation (Rama Rao et al., 1982). Many workers have reported that peroxidase and IAA oxidase activities are associated with the same protein molecule, but they have different active sites or involve differential allosteric activation (Siegel and Galston, 1967; Raa, 1973). In contrast, "auxin protectors" are a class of plant growth substances found in all dicotyledonous plants investigated so far. The term was originally coined (Stonier and Yoneda, 1967) to describe naturally occurring substances which inhibit the peroxidase-catalysed oxidation of indole acetic acid (IAA).

Merremia emarginata is a unique plant with stretchable internodes even at later stages of plant age, thus providing an important tool to study the process of cell elongation and its regulation. Using this model system, endogenous levels of plant growth hormones were also studied (Patel and Thaker, 2007). The role of wall-loosening enzymes (Patel and Thaker, 2004a) as well as the changes in wall components (Patel and Thaker, 2004b) have been reported after exogenous application of hormones. It was observed that although there was no significant effect on growth, wall-

yielding properties increased with internode growth. Here we report on the changes in wall stiffening system including peroxidase and IAA oxidase activities together with the changes in phenols and IAA protecting system to understand the mechanisms of elongation in *Merremia emarginata*.

MATERIALS AND METHODS

Collection of the plant material

The experimental plants were grown in the botanical garden of the Department of Biosciences, Saurashtra University, Rajkot. Seedlings of *Merremia* were transferred to small pits in continuous rows at a distance of 1 ft. These plants were grown up to flowering (nearly 30 days).

Preparation of the plant material

The internodes were separated from each other at the center of the connecting nodes. The age of the internodes was considered according to its position from the apex. The ones at the apex, next to the primordia, were considered to be the youngest and the internodes near to the root as mature. The young internodes i.e. No 1 through No 5 were considered individually while internode No. 6-7-8; 9-10-11; 12-13-14 and ≥15 were considered as a pooled group because of their similarity in biochemical and other characteristics studied earlier in pilot experiments. At least 10-15 replicates in each of these 9 groups were taken for the experimental analysis to eliminate the probable variations due to biological differences among individual plants.

Hormone treatments applied to the internodes

Three hormones were selected for the treatment of the internodes: gibberellic acid (GA_3), phenyl acetic acid (PAA) and naphthyl acetic acid (NAA). They were applied at concentrations 100 ppm, 75 ppm and 10 ppm. All hormones were obtained from Hi- media Co. Bombay, INDIA.

The internodes from nine groups kept in 20 mM, K-PO_4 buffer, pH 6.4,

with /or without hormone (each one prepared in 20 mM, K-PO_4 buffer, pH 6.4), were thoroughly submerged in wide-bottom, glass containers. The internodes were incubated with the different solutions for 16 h under continuous light (1000 Lux) at room temperature (25±3 °C).

Growth analysis

Length of individual internodes, their fresh and dry weights, were considered for growth analysis. The internodes were thoroughly washed and blotted on filter paper to remove the adhering water. The length was considered as an "average" of all representatives in the respective group. The mean value of 25-30 was calculated with ± standard deviation.

Preparation of the enzyme extract

The cytoplasmic and wal-bound enzyme extracts were prepared as described in Patel and Thaker (2004a).

Peroxidase activity

Peroxidase activity in cytoplasmic and wall-bound enzyme extracts was estimated by the method of Thaker (1998). The change in the optical density was recorded at 400 nm for chlorogenic acid and caffeic acid and at 470 nm for guaiacol. The activity was expressed as ΔA_{400} mg $P^{-1}min^{-1}$ and ΔA_{470} mg $P^{-1}min^{-1}$, respectively.

IAA oxidase activity

For determination of IAA oxidase activity, a modified method of Gordon and Weber (1951) was used. The change in color was measured at 540 nm after 20 min. The calibration was done using IAA and the cytoplasmic and wall-bound IAA oxidase activity was expressed as µg IAA oxidized mg P^{-1} min^{-1}.

Estimation of the phenolic compounds

Internodes from different age groups and treatments were weighed and ground in 80% ethyl alcohol containing 200 mM borate buffer (pH 7.6) in the dark and incubated overnight. The mono-, di- and total phenolic compounds were extracted as described by Pansuria et al. (2006).

IAA protecting assay

The extract prepared for the estimation of phenol contents was used as the source of IAA protectors. $MnCl_2$ (1 mM), dichlorophenol (1 mM), 20 mM phosphate buffer (pH 6.4) and Horseradish Peroxidase (HRP, 20 µg/ml), 1 m; each were added to the extract (1.0 ml). The reaction was initiated by the addition of 1 ml of IAA (200 µg/ml).

The degradation of IAA was determined at different time intervals by removing 1.0 ml of the reaction mixture followed by the addition of a double volume of Salkowaski reagent and measured as described for IAA oxidase. The magnitude of IAA protection was then calculated from a standard curve prepared by varying concentrations of IAA (20-200 µg) to monitor the degradation of IAA following the lag caused by the protectors in the presence of HRP and expressed as lag produced in minutes per internode. All measurements were done in triplicates. The results presented are mean values ± SD.

Protein Estimation

Protein content was estimated following the method of Bradford (1976).

Statistical Analysis

For each analysis five replicates were taken and mean values ± standard deviations were presented. Inhibition by each hormone was statistically processed using ANOVA.

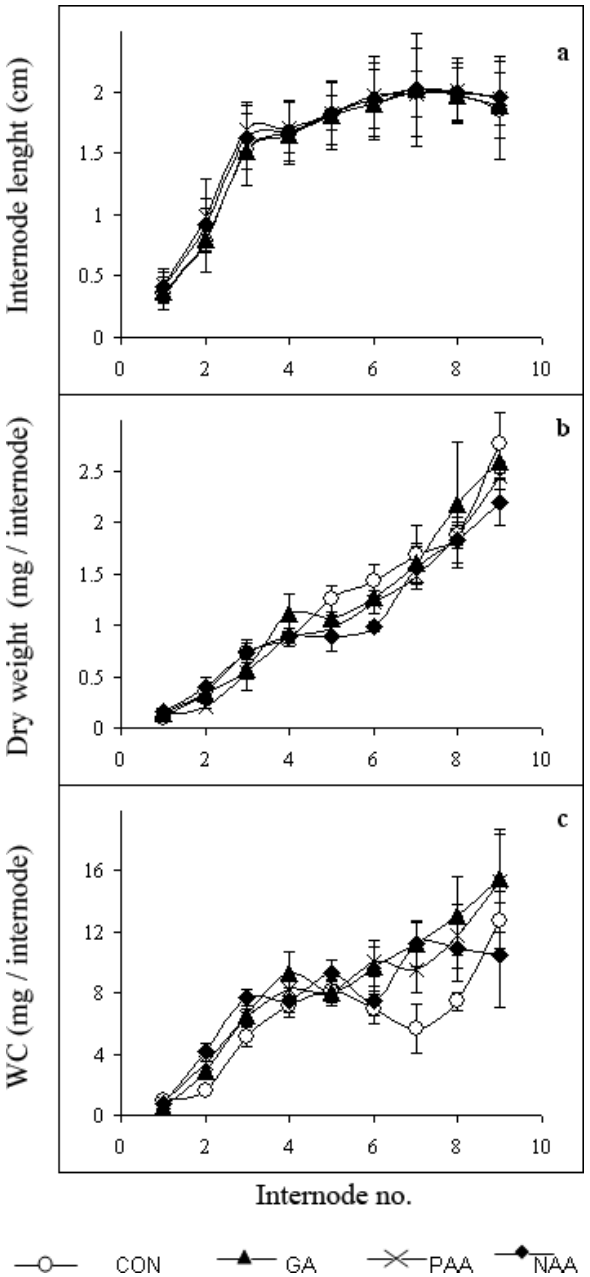

Fig. 1. Changes with age in length (a), dry weight (b) and water content (c) in hormone-treated internodes of *Merremia emarginata*. Vertical bars represent SD.

RESULTS AND DISCUSSION

The analysis of internode length, dry weight and water content (Fig. 1a,b and c) showed that the effect of hormones on the growth parameters was statistically non-significant in ANOVA (Analysis of variance). This result suggested that exogenous application of hormones did not affect internode growth parameters. Water content plays an important role in the process of cell elongation and influence of hormones on water uptake has been reported (Taiz, 1984). However, the lack of such a relation in the present study suggests that *Merremia emarginata* has optimum levels of endogenous hormones.

Auxin in plants can exist as (i) free that is readily available for growth (ii) conjugated form which is released by cells as and when required and (iii) oxidized form which cannot be used in growth. The latter is regulated by IAA oxidase and peroxidase system and the counteraction of the auxin protecting system. In our earlier studies it was observed that at the time of internode elongation free auxin content was low and it increased with the decrease in the rate of elongation. In contrast, conjugated IAA showed a declining trend where free IAA content was remarkably high, suggesting that conjugated IAA might have been mobilized during the later phase of internode development (Patel and Thaker, 2007).

Peroxidase restricts growth by rigidifying the cell wall by covalently cross-linking the phenolic dimmers (Fry, 1986). Changes in cytoplasmic and wall-bound peroxidase activity using different substrates are presented in Fig. 2. When peroxidase was assayed with caffeic acid as a substrate, in GA_3-treated internodes the activity remained low up to the 6th internode and it increased at a later stage while in NAA or PAA-treated internodes the activity was higher or near to the control values. In the wall-bound fraction GA_3-treated internode showed low activities whereas in NAA and PAA-treated internode values were initially low and remained near to the control in the subsequent period.

In chlorogenic acid-specific peroxidase assay the activities remained higher in hormonal-treated internodes even after GA_3 treatment, except for the initial two internodes, both in cytoplasmic and wall-bound fractions (Fig. 2b, 2e).

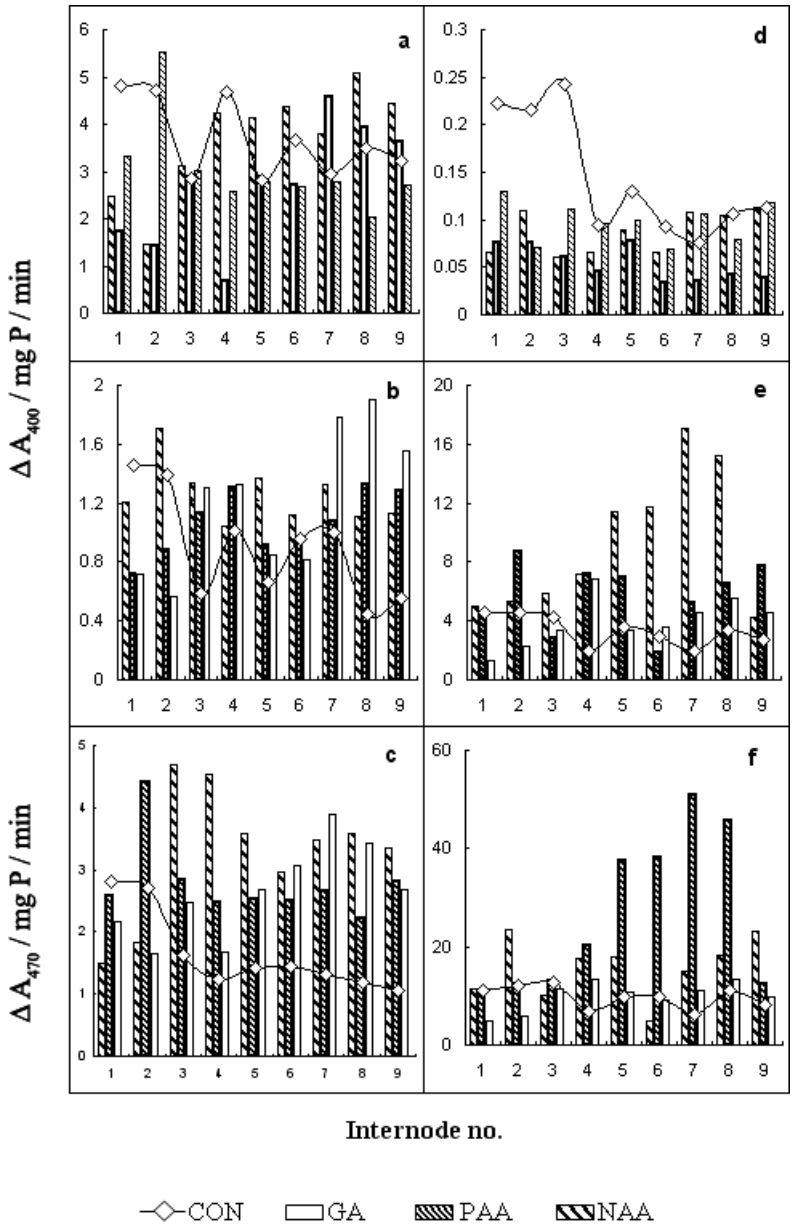

Fig. 2. Changes with age in cytoplasmic (a,c,e) and wall bound (b,d.f) peroxidase activities: caffeic acid (a,b), chlorogenic acid (c,d) and guaiacol(e,f) in internodes of *Merremia emarginata*. Vertical bars represent SD.

Changes in peroxidase activity assayed with guaiacol as a substrate are presented in Fig. 2 (c, f). In the cytoplasmic fraction, all hormones showed marked promotion in the activity as compared to the control. In NAA treatment the activities were higher than PAA.

In general, wall-bound activity was promoted under NAA treatment when assayed with guaiacol or chlorogenic acid as substrates. Further, in the cytoplasmic fraction, when assayed with chlorogenic acid and guaiacol, NAA and PAA showed promotion in the activity. On the other hand, when caffeic acid was used as a substrate, the activity was inhibited by GA treatment, in both the cytoplasmic and wall-bound fractions though more prominently in the wall-bound fraction during the elongation phase. It has been reported that exogenous auxin stimulates IAA oxidase and peroxidase activities (Henry et al., 1971).

An inverse correlation between the activities of these enzymes and endogenous IAA content and/or elongation growth has been reported (Jupe and Scott, 1989; Zheng and Van Huystee, 1992). A role for peroxidases in primary cell wall stiffening is generally accepted (Fry, 1986). Reduced growth associated with higher peroxidase activity has been tentatively explained in terms of the capacity of peroxidase to degrade IAA (Liang et al., 1977).

The changes in IAA oxidase activity are presented in Figure 3. In the cytoplasmic fraction IAA oxidase activity was higher up to the 3rd internode and declined thereafter both in control and treated internodes (Fig. 3a). In contrast, in the wall-bound fraction, an inhibition in activities was observed in GA_3 treatment. In NAA and PAA-treated internodes the values remained higher than the control (Fig. 3b).

In control internodes, peroxidase and IAA oxidase activities were higher during the elongation period and comparatively less activities were observed in the later stages. Bagatharia and Chanda (1998) concluded that peroxidase restricted the elongation growth of mung beans by allowing the formation of diphenyl cross-links while IAA oxidase might play a role in the fine regulation of IAA cellular levels. However, in the present study, no such correlation was evident, although with GA_3 treatment the activity remained at low levels as compared to the control. A role of GA in the inhibition of both IAA oxidase and peroxidase activities is well documented

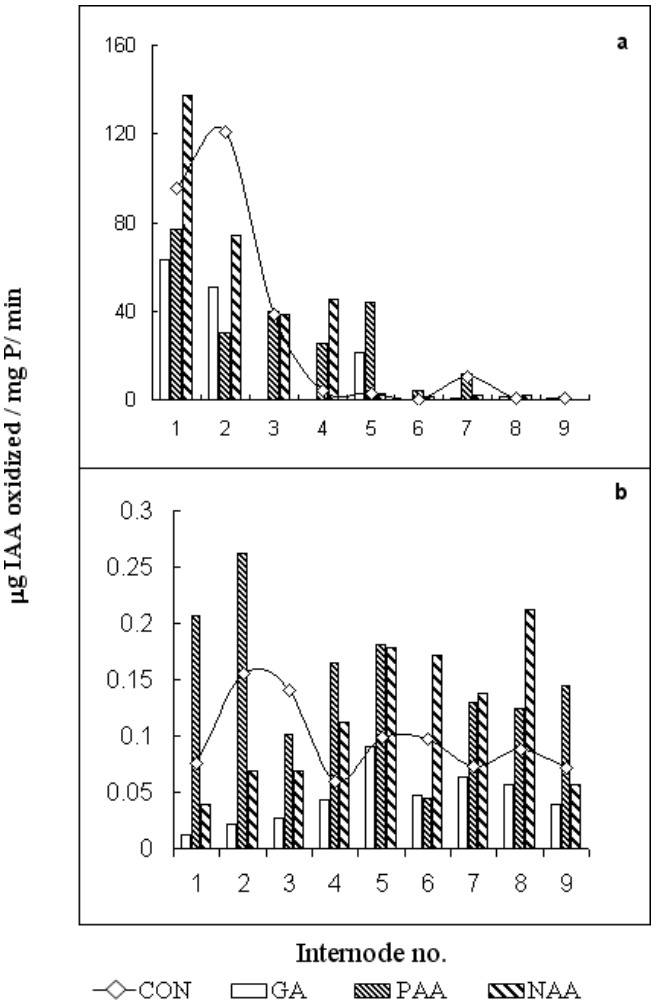

Fig. 3. Changes with age in cytoplasmic (a) and wall bound (b) IAA oxidase activity in internodes of *Merremia emarginata*. Vertical bars represent SD.

(Saroop et al., 1987).

The monophenol content in the control and hormone-treated internodes is presented in Figure 4a. Higher levels of monophenols in almost all stages of the internode development were observed in the control. In the initial stages, the GA_3-treated internodes had elevated levels of monophenols as compared to other treatments, although among NAA and PAA, the latter

showed slightly higher values up to the 2nd internode while the former had more monophenols in the later stages i.e. 6th internode onwards, which in turn either paralleled to GA_3-treated internodes or exceeded them.

In the initial stages the values for the dihydroxy phenols were comparable to the control in all treatments except for the slightly higher content in the GA_3-treated internodes (Fig. 4b). In the later stages i.e. 6th internode onwards, the PAA and NAA treated internodes showed either equal levels to the control or a slight increase in the NAA-treated ones, while GA_3-treated internodes contained remarkably high content of dihydroxy phenols as compared to the control samples.

Our results showed a continuous increase in total phenols in all hormone treatments while the control showed an increase at the initial stages i.e. up to 3rd internode followed by a decreased in the later stages (Fig. 4c). A marked increase was observed in NAA-treated internodes, but the GA_3 and PAA-treated internodes showed parallel values.

Though there is little evidence that phenolics play a key role in plant growth and development, many phenolics affect growth when applied to plant tissue (Vickery and Vickery, 1981). Some phenolics have been shown to interact directly with plant hormones or to affect enzyme activities and cause changes in hormonal balance (Harbone, 1980). In genera, it has been observed that monophenols stimulate IAA oxidase activity and inhibit growth while diphenols and polyphenols counteract IAA destruction and promote growth. In the present study, an inverse correlation between phenolic content and internode growth was observed with diphenols and total phenols while monophenol levels remained higher during elongation growth of internodes in the control and marked inhibition was observed in all treatments with PGRs tested (Fig 4b). Further, the activity of peroxidase assayed with different substrates did not show any clear correlation with phenolic content, suggesting thereby that regulation of substrate concentration and enzyme protein may control the internode growth.

IAA protecting assay

Stonier et al. (1979) reported that that most of the protector activity in sunflower was associated with chlorogenic acid as determined by means of

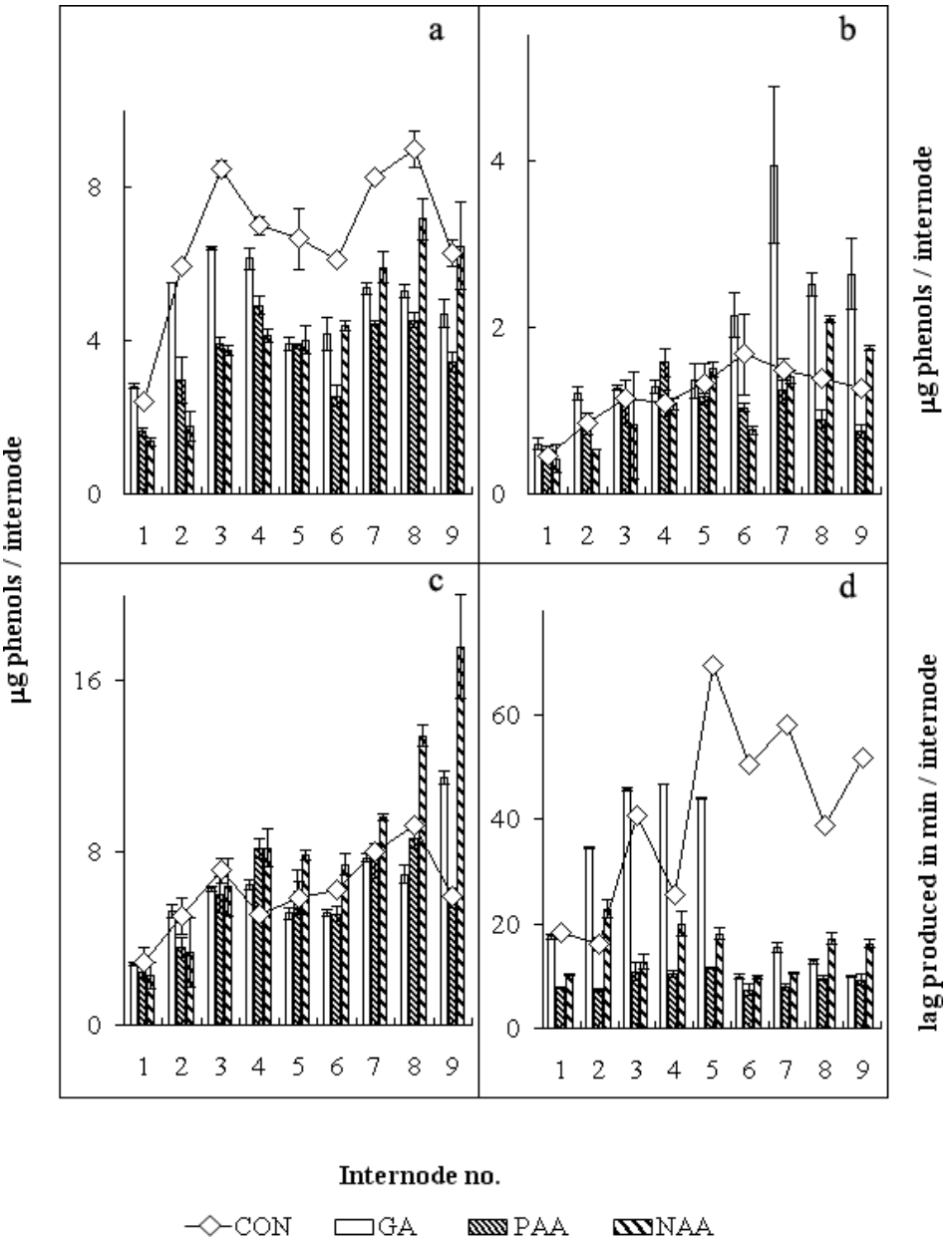

Fig. 4. Changes with age in monohydroxy (a) and dihydroxy (b) total phenols (c), and IAA protecting assay (d) in internodes of *Merremia emarginata*. Vertical bars represent SD.

chromatography, UV spectra and acid hydrolysis. Some protector activity was associated also with a derivative of chlorogenic acid tentatively identified as isochlorogenic acid. The protectors identified by them seemed to be derivatives, polymers or complexes of the o-dihydroxy propanoids (Novak and Galston, 1971; Stonier, 1976).

In the present study, the results of IAA protecting assay showed that the level of protection in the control internodes was lower at the initial stages (Fig. 4d). Among the hormone-treated ones, GA_3-treated internodes showed a slightly increasing trend with age during the elongation period while in PAA-treated internodes the protectors were found to be almost at similar levels throughout all stages though at higher levels as compared to NAA. However, in the later stages remarkably higher values were obtained for the control as compared to all the hormone treatments.

CONCLUSION

An inverse correlation between di- and total phenolic content and growth was observed supporting the general observation that monophenols stimulate IAA oxidase activity and inhibit growth while diphenols and polyphenols counteract IAA destruction and promote growth. Monophenols remained higher during elongation in control internodes and they were markedly inhibited in all hormone treatments, suggesting that hormones may promote growth via controlling monophenol content. Any clear relationship between phenolic content and peroxidase activity assayed with different hydrogen donors was not observed, suggesting that regulation of substrate concentrations and enzyme protein may control internode growth. Hormones may affect internode growth via decreasing the levels of IAA oxidase activity as indicated by the low levels of IAA oxidase in hormone-treated and especially GA_3-treated internodes.

References

Bagatharia, S. B., S.V. Chanda, 1998. Changes in peroxidase and IAA oxidase activities during cell elongation in Phaseolus hypocotyls. Acta Physiol. Plant., 20, 9-13.

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.

Fry, S.C., 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu. Rev. Plant Physiol., 37, 165-186.

Gordon, S.A., R. P. Weber, 1951. Colorimetric estimation of indole acetic acid. Plant Physiol., 26, 192-195.

Harbone, J.B., 1980. Plant phenolics. In: Secondary plant products. EA Bell, BV Charlwood (ed), Springer-Verlag Berlin, vol. 8, 329-402.

Henry, E.W., J.G. Valdovinous, T. E., Jensen, 1971. Ethylene enhances peroxidase in abscission cells of tobacco flower pedicels. Plant Physiol. 47, 83.

Jupe, S.C., I. M. Scott, 1989. The slender phenotype of pea: stem growth, peroxidase levels and ethylene responses. Physiol. Plant., 77,59-66.

Liang, G.H., K.C. Lee, K. Chung, Y.T. Liang, B.A. Cunningham, 1977. Regulation of internode length by peroxidase enzymes in grain sorghum. Theor. Appl. Gen. 50, 137-147.

Novak, J., A. W. Galston, 1971. Studies on auxin protector substances, IAA-oxidase and peroxidase in cotyledons of *Pharbitis nil*. Plant and Cell Physiol., 12, 931-940.

Pansuria, S. H., R. Chudasama, S. B. Bagatharia, V.S. Thaker, 2006. Production of diphenol and monophenol compounds from the leaf callus of Morus alba and studies on their antibacterial activity. Ind. J. Plant Physiol., 11, 36-40.

Patel, D., V. S. Thaker, 2004a. Studies on cell wall loosening enzymes in hormone treated internodes of *Merremia emarginata*. Acta Physiol. Plant., 26, 131-138.

Patel, D., V. S. Thaker, 2004b. Turnover of cell wall components during internode growth in *Merremia emarginata*. Acta Physiol. Plant., 26, 139- 146.

Patel, D., V. S. Thaker, 2007. Estimation of endogenous contents of phytohormones during internode elongation in *Merremia emarginata*. Biol. Plant., 51, 75-79.

Raa, J., 1973. Cytochemical localization of peroxidase in plant cells. Physiol. Plant., 28, 132-133.

Rama Rao, N., S. C. Naithani, R.T. Jasdanwala, Y.D. Singh, 1982. Changes in indole-acetic acid oxidase and peroxidase activities during cotton fiber development. II. Pflanzen. Physiol., 106, 157-165.

Saroop S., V.S. Thaker, P.P. Vaishnav, Y. D. Singh, 1987. Changes in peroxidase activity as affected by gibberellic acid and cycocel in cucumber seedlings. Acta Biol. Szeged., 33, 17-24.

Schopfer, P., 1996. Hydrogen peroxide mediated cell wall stiffening *in vitro* in maize coleoptiles. Planta, 199, 43-49.

Showalter, A. M., 1993. Structure and Function of plant cell wall proteins. The Plant Cell, 5, 9-23.

Siegel, B. Z., A. W. Galston, 1967. Indole acetic acid oxidase activity of apoperoxidase. Sci., 157, 1557-1559.

Stonier, T., 1976. In: collected abstracts of the paper demonstrations. PE Pilet (ed), The ninth international conference of plant growth substances. Lausanne (Switzerland).

Stonier, T., Y. Yoneda,1967. Stem internode elongation in the Japanese morning glory (Pharbitis nil choisy) in relation to an inhibitor system of auxin destruction. Physiol. Plant., 20, 13-19.

Stonier, T., S. Stasinos, K. B. S. MurthyReddy, 1979. The masking of peroxidase catalyzed oxidation of IAA in *Vigna*. Phytochemistry, 18, 25-28.

Taiz, L., 1984. Plant cell expansion:regulation of cell wall mechanical properties. Annu. Rev. Plant Physiol., 35, 585-657.

Thaker, V. S., 1998. Role of peroxidase and glycosidases in regulation of sink size in developing seeds of *Hibiscus esculentum*. Acta Physiol. Plant., 20, 179-182.

Vickery, M. L., B. Vickery, 1981. Secondary plant metabolism. The Macmillan press Ltd, London, ISBN 0-333-27017-7.

Zheng, X., R. B. Van Huystee, 1992. Peroxidase-regulated elongation of segments from peanut hypocotyls. Plant Sci., 81, 47-56.