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EFFECT OF WOUNDING AND STRESS ON PROTEINASE INHIBITOR PROFILE OF *Zizyphus jujuba* LEAVES

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ABSTRACT

Effect of wounding and stress during the growth phase of *Zizyphus jujuba* showed quantitative changes in proteinase inhibitor (PI) pattern in the leaves. The PI level was found to increase when 30 days old plants were artificially wounded. The PI was found to be maximum at 16 hr after wounding, which was simultaneously followed by an increase in total protein content in leaves of *Z. jujuba*. Similarly, the PI level in the leaves was also found to increase when the plants were subjected to high osmotic (NaCl and mannitol) and drought stress treatment. It was also observed that the progressive increase in the osmotic stress led to the accumulation of proline in the leaves.

Keywords: *Zizyphus*, proteinase inhibitor, wounding, drought stress and osmotic stress.

INTRUDOCTION

Plants in general are exposed to a number of abiotic stress such as drought, temperature, salinity, alkalinity, water logging and nutrition which limit their distribution and productivity[1]. Plants are sessile and have therefore developed mechanisms to adapt to a variety of environmental stresses in order to survive[2].

The genus *Zizyphus* belongs to the buckthorn family (*Rhamnaceae*). It is a genus of about 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions in the world[3]. Compared to other more commonly cultivated fruit tree species, *Zizyphus* species have several physiological and morphological characteristics that may contribute to their ability to adapt to arid environments. These trees are well adapted to seasonal drought and hot conditions[4]. Our earlier studies have shown that many parts of *Z. jujuba* are an excellent source of PI[5]. PIs comprise one of the most abundant classes of proteins in plants. The function of these inhibitors is to control proteolysis within cells, organelles or fluids when limited proteolysis is important for the biochemical or physiological process[6].

Wounding and abiotic stress results in significant morphological and metabolic changes which limit the crop yield and quality. A variety of genes have been described in plants that are expressed during wounding and environmental stresses. A number of small molecular weight wound induced PIs have been purified from the leaves of tobacco (*Nicotiana tabacum*)[7]. A significant increase in PI level was also observed in sweet potato when subjected to artificial wounding[8]. Stress has also induced synthesis of number of polypeptides in *Lathyrus sativus*[9].

Z. jujuba being a store house of PI and a widely available plant, offers an ideal system for studying changes in PI during abiotic stress and wounding. The present study has been undertaken to observe the effect of stress and wounding on PI level of *Z. jujuba.*

MATERIAL AND METHODS

Chemicals

Trypsin, a-chymotrypsin (3 x crystallized from bovine pancreas), casein (vitamin free) and N-acetyl L- tyrosine ethyl ester (ATEE) were obtained from Sigma Chem. Co. (St. Louis, M O) U.S.A. All other chemicals were of analytical grade.

Germination condition

Z. jujuba seeds were obtained from dried fruits of the single tree available in the orchard of Post Graduate Teaching Department of Biochemistry, Nagpur University, Nagpur. The seeds obtained from the fruits were imbibed in

distilled water for 24 hr. The seeds were then surface sterlized with 1 mM mercuric chloride for 15 min. and rinsed thoroughly with sterile distilled water to remove all the traces of mercuric chloride. The swollen seeds were sown in plastic bags filled with fine sterlized sand and watered to field capacity. Plants were grown under controlled environmental conditions at a photoperiod L:D = 11:13. The plants were watered daily with tap water and thrice a week with half strength Hoagland solution as nutrient medium[10]. The plants were grown in a controlled environment to prevent damage from natural pests and insects.

Drought stress treatment

Thirty days old plants were subjected to progressive water stress by withholding the water supply for 6, 8, 10, 12, 14, and 16 days. Control plants were maintained at field capacity by watering the plants every day at same time.

Osmotic stress treatment

For high osmotic treatment, 30 days old plants were watered twice at field capacity with 100 mM, 200 mM, 400mM and 600 mM NaCl and 2.5%, 5%, 10% and 20% mannitol in half strength Hoagland solution. The control plants were watered with only half strength Hoagland solution. Leaves were harvested after each stress treatment and assayed for proteinase (trypsin and chymotrypsin) inhibitor activity.

Development of artificial wound

Artificial wound was developed by carefully subjecting a mature leaf to heat treatment for few seconds[11,12]. The leaves closer to the wounded leaf were harvested after every 4, 8, 12, 16, 24 and 48 hr and assayed for proteinase inhibitor activity. The control plants were raised under same conditions except that no wound was induced artificially in them.

Extraction of PI

Ten percent extract of leaves was prepared in 0.2 M sodium phosphate buffer (pH 7.6) containing 0.9% NaCl (PBS) by method of Varghese and Patil[5]. The extract was then filtered and centrifuged at 12,000 rev/min. (9668 x g) in C-24 Remi refrigerated centrifuge for 20 min. The supernatant obtained was subjected to 90% ammonium sulphate precipitation for the complete precipitation of proteins[13]. The precipitated proteins were collected by centrifugation at 12,000 rev/min. (9668 x g). The precipitate was dissolved in minimum amount of PBS and kept for dialysis overnight under cold condition against large volume of same buffer. The dialysed sample so obtained was used for the determination of PI content.

Proteinase inhibitor assay

Trypsin inhibitory activity was assayed by the method of Kakade *et al*. (1969) using casein as the substrate[14]. One unit of trypsin is defined as the amount of enzyme which liberates 100 mg of peptide fragments under experimental conditions. One trypsin inhibitor unit is defined as that amount of inhibitor which inhibits the liberation of 100 mg of peptide fragments by trypsin under experimental conditions. The inhibitory activity towards chymotrypsin was determined by the method of Prabhu and Pattabhiraman (1977), using ATEE as the substrate[15]. One unit of chymotrypsin is defined as the amount of enzyme which liberates 100 mg N-acetyl L-tyrosine under experimental conditions. One chymotrypsin inhibitor unit is defined as the amount of inhibitor which inhibits the liberation of 100 mg of N-acetyl L-tyrosine under the experimental conditions.

Protein content was determined by the method of Lowry *et al.* (1951), using BSA as the standard[16].

Estimation of Proline content

Proline content of the leaves was estimated by ninhydrin reagent method[17].

Statistical analysis

Duplicate samples were collected at each sampling frequency. Three replications were conducted through out the experiments and the results presented in the Tables 1-4 were processed following single linear regression equation.

RESULTS AND DISCUSSION

Artificial wounding in the leaves of *Z. jujuba* induced considerable synthesis of inhibitor protein as compared to the control group (Table -1). Wound induced synthesis of secondary metabolites has been reported in many crop plants as an immediate response. There was a sharp increase in TI from 8-16 hr which further decreased untill 48 hrs. Similar reports were also reported by Sasikaran *et al*. in sweet potato leaves when subjected to artificial wounding[8]. A similar pattern in chymotrypsin inhibitor content was also observed in the leaves. It is a known fact that an artificial wound can mimic the action of an insect bite and stimulate the production of the inhibitor[11,12]. The increase in PI level in response to wounding in *Z. jujuba* leaves suggest the role of PI as potential

Table - 1 : Effect of wounding on PI level and protein content in *Z. jujuba* leaves

Time (hr)	PIU/g TIU/g	PIU/g CIU/g	Protein mg/g
Control	1250	625	2.2
4	1875	937	4.0
5	3125	1560	4.5
12	5000	2500	4.7
16	5625	2812	5.5
24	3125	1562	6.5
48	625	312	7.0

Table - 2 : Effect of drought stress on PI level and protein content in *Z. jujuba* leaves

Water Stress (days)	PIU/g TIU/g	PIU/g CIU/g	Protein mg/g
Control	1250	625	2.2
6	2500	1200	2.0
8	3125	1562	1.7
10	3750	1875	1.5
12	3125	1562	1.5
14	1875	937	1.2
16	625	312	1.0

Table-3 : Effect of salt (NaCl) stress on PI level, protein and proline content in *Z. jujuba* leaves.

NaCl (mM)	PIU/g TIU/g	PIU/g CIU/g	Protein mg/g	Proline mg/g
Control	1250	625	17.7	0.39
100	2500	1250	14.2	1.14
200	3125	1500	12.5	1.27
400	1875	935	10.2	1.27
600	625	300	7.7	1.28

Table - 4 : Effect of mannitol stress on PI level, protein and proline content in *Z. jujuba* leaves

Mannitol (%)	PIU/g TIU/g	PIU/g CIU/g	Protein mg/g	Proline mg/g
Control	1875	940	15.0	0.39
2.5	5625	2812	13.7	0.39
5	6250	3125	12.7	0.72
10	4375	2187	10.2	1.16
20	3750	1800	7.2	1.24

PIU= Proteinase inhibitor units; TIU= Trypsin inhibitor units; CIU = Chymotrypsin inhibitor units

defense proteins against pests and pathogen. The serine PI have been studied in detail as inducible defense related proteins[18,19]. The induction in defense responses is particularly interesting since it can occur both at the local site of injury and systemically. The PIs are induced during wounding and herbivore and influence herbivore performance by inhibiting insect digestive enzymes[20-23].

Many physiological and biochemical processes are even sensitive to changes in water balance. An increase in PI (trypsin and chymotrypsin) in the plants subjected to progressive drought stress (6-16 days) is presented in Table-2. A gradual increase in PI content was observed in the leaves when the water capacity was withheld for 10 days, but there was a decline in the total protein content when the plants were subjected to severe drought stress by withholding the water capacity for 16 days. The PI content then started decreasing in the leaves with a significant morphological change in the overall plant. There are numerable examples in the literature indicating the synthesis of certain proteins in response to drought stress in many plants. Sharma and Singh (2003) have reported the synthesis of cyclophilin like protein in leaves and seeds of sorghum[24]. A glycine rich protein was reported to be synthesised in response to water stress in maize plant[25]. However, to our knowledge, a relationship between drought stress tolerance and PI level has not been studied in *Zizyphus* species yet. *Z. jujuba* being a drought tolerant plant, the increased PI level in response to water stress is possibly due to the involvement of PI as a stress protein.

The effect of osmotic stress (NaCl and Mannitol) is presented in table 3 and 4. The leaves of the plants harvested after 7 days of NaCl stress showed an increase in PI upto 200 mM NaCl, which then decreased gradually with further increase in salt stress. Similarly the PI in leaves of the plant subjected to progressive mannitol stress increased till 5% concentration after which it started declining. There was also a parallel decline in total protein content with progressive NaCl and mannitol stress. Similar results have been reported by Downing (1992), where the BnD22 gene expression in *Brassica napus* leaves in response to salt stress contains the signature motif of soybean kunitz trypsin inhibitor[26]. The increase in PI in response to osmotic stress in *Z. jujuba* probably confers its role as stress protein helping the plant to survive under high osmotic conditions.

High osmoticity even led to the high accumulation of proline in the leaves (Table 4,5). There have been reports on proline accumulation in transgenic tobacco plant with an over expression of a salt stress protein osmotin[27,28]). The increase in proline accumulation in *Z. jujuba* leaves in response to osmotic stress may be to confer tolerance to osmotic stress. Proline is reported to act as an osmoprotectant by raising the internal osmotic pressure, which protects the cell against osmotic stress[29].

From the above results, it appears that the PI of *Z. jujuba* being an enzyme inhibitor, is also a stress protein which imparts tolerance in the plant against abiotic stress. The gene of PI once identified and cloned can be profitably cultivated in stress prone areas.

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REFERENCES

1. Renu, K.C. and Suresh, K.S. *Curr. Sci.* **74,** 25-34 (1998)
2. Abdin, M.Z., Rehman, M., Israr, M., Srivastava, P.S. and Bansal, K.C. *Ind. J. Biotechnol.* **1**, 225-244 (2001)
3. Johnston, M.C. *Am. J. Bot.* **50**,1020-1027 (1963)
4. Jawanda, J.S. and Bal, J.S. *Ind. Hortic* Oct-Dec. 19-21 (1978)
5. Varghese, J. and Patil, M.B. *Nag. Univ. Sci. J.* **6**, 132-158 (2005)
6. Ussuf, K.K., Laxmi, N.H. and Mitra, R. *Curr. Sci.* **80**, 847-853 (2001)
7. Pearce, G. Johnson, S. and Ryan, C.A. *Plant Physiol.* **102** (2), 639-644 (1993)
8. Sasikaran, K., Rekha, M.R. and Padmaja, G. *Bot. Bull. Acad. Sin.* **43**, 291-298 (2002)
9. Sinha, K.M., Sachdev, A., Johari, R.P. and Mehta, S.L. *J. Plant Biochem. Biotechnol.* **8**, 47-51 (1999)
10. Hoagland, D.T. and Arnon, D.I. *Univ. Calif. Expt. Stn. Grc*. **347,** 1 (1938)
11. Green, J.R. and Ryan, C.A. *Sci.* **175**, 776-777 (1972)
12. Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F. and Boulter, D. *Nature* **330**, 160-163 (1987)
13. Jayaraman, J. Laboratory, Mannual, Wiley Eastern Limited, New Delhi 82-84 (1992)
14. Kakade, M.L., Simons, N and Liener, I.E. *Cer. Chem.* **46**, 518-526 (1969)
15. Prabhu, K.S. and Pattabhiraman, T.N. *Ind. J. Biochem. Biophys.* **14**, 96-98 (1977)
16. Lowry, O.H., Rosebrough, N.J. Farr, A.L. Randall, R.J. *J. Biol. Chem.* **193**, 265-275 (1951)
17. Chiddharwar, M.K. and Patil, M.B. Charaterization and Biochemical studies on *Rhizobium* of cowpea, Ph.D. Thesis, Nagpur University, Nagpur, India (1997)
18. Ryan, C.A. The Biochemistry of Plants, New York : Academic **6**, 351-370 (1981)
19. Laskowski. M. Nutritional and Toxicological significance of Enzyme Inhibitors in Foods. New York, 1-17 (1986)
20. Koiwa, H. Bressen, R.A., Hasegaw, P.M. *Trends Plant Sci.* **2**, 379-384 (1997)
21. Tamayo, M.C., Rufat, M., Bravo, J.M. and San-Segundo, B. *Planta* **211,** 62-71 (2000)
22. Edwards, P.J., Wratten, S.D. and Cox, H. *Ecol. Entomol.* **10**, 155 (1985)
23. Broadway, R.M., Duffey, S.S. Pearce, G. and Ryan, C.A. *Entomol. Exp. Appl.* **41**, 33-38 (1986)
24. Sharma, A.D. and Singh, P. *J. Plant Bichem. Biotechnol.* **12**, 77-80 (2003)
25. Gomez, J., Martinez, S.D., Stiefel, V. Rigau, J., Pere, P., and Pages, M. *Nature* **334**, 262-264 (1988)
26. Downing W.L. *Plant J*. **2** 685-693 (1992)
27. Singh, N.K. *Plant Physiol*. **79**, 126-137 (1985)
28. Barthaker, S.J. *Plant Biochem. Biotechnol.* **10**, 31-37 (2001)
29. Csonka, L.N. and Hanson, A.D., *Annu. Rev. Plant Physiol.* **45**, 569-606 (1991)