International Journal of Genetic Engineering and Biotechnology.
ISSN 0974 3073 Volume 5, Number 2 (2014), pp. 115-120

http://www.irphouse.com

Cytokinins Initiating Protective Reactions in Water Stressed Chickpea Root Nodules

Virbala Sharma and Kamal Jit Singh*

Department of Botany, Panjab University, Chandigarh-160014, India.

Abstract

Role of exogenous application of cytokinins was assessed in plant's defense against water stressed chickpea (*Cicer arietinum* L.) root nodules. Endogenous titre of the nodular phenolic compounds suggests that cytokinins act synergistically during water scarcity in inducing phenol-biosynthesis pathways and maintaining symbiotic relationship of the legume crop. Cytokinins play an important role in nodular metabolism by antagonizing the deleterious effects of water stress and the accumulation levels of individual phenolic compounds determine tolerance level of a genotype. Salicylic acid participates in initiation of protective reactions of the host plant and *p*-Hydroxybenzoic acid and *p*-Coumaric acid determines the tolerance and susceptibility levels of a genotype, respectively.
Keywords: Phenolics, Drought, Stress, Cytokinin, Chromatography.

Introduction

Chickpea (*Cicer arietinum* L.) an important legume crop in the semi-arid tropics, is relatively a drought tolerant crop. Its larger tap root system allows maximum uptake of the ground water. Water stress affects the plant in different ways, although it adjusts to mild deficits by reducing its water losses and increasing its uptake. Plant's defense against biotic and abiotic stresses is mediated through various signaling pathways leading to the production of many proteins and non-protein compounds (Maffei *et al*., 2007; Vicent and Plasencia, 2011). Cytokinins antagonize stress induced changes such as delayed senescence (Catsky *et al*., 1996). Plant hormones are the main signals from root to shoot communications and vice-versa (Davies, 1995 and Naqvi, 1995). ROS-oxidative burst is the immediate response to regulate gene expression associated with defense mechanisms (Kawano, 2003). Effect of exogenous applications of cytokinins (6-BAP) was studied in an ameliorative role in minimizing water deficit induced stress in the form of phenolics participating in protective reactions of root nodules in chickpea plants.

Materials and Methods

Two released varieties of chickpea (*Cicer arietinum* L.) namely H208 (stress tolerant) and H96-99 (susceptible) were procured from CCS HAU, Hisar (Haryana). The experiments were conducted in open growth houses in earthenware pots filled with sieved garden loam, sand and farm yard manure in 1:1:1 ratio. Tap water was used for irrigation purposes. Seeds were inoculated with standard rhizobial strain obtained from IARI, New Delhi. Moisture content of soil was checked regularly. Both the crops were divided into three lots of 40 pots each. At 80 DAS stage, one lot of each crop was subjected to 5 days of water deficit by withholding water irrigation. Exogenous application of cytokinin (6-BAP) leaf spray was made twice with a gap of 24 h before withholding water. Third lot of each crop served as control.

Reverse-phase chromatography (Waters, HPLC) was used for the optimum separation of phenolic acids in multistep gradient using solvent (A) Acetic acid–Water (2:98): (B) Butanol-Methanol (8:92) following the method of Hahn *et al*. (1983). Gradients of A and B solvents with a ratio 95:5 to 50:50 was used in a programmed run of 45 min. Separation was performed by isocratic program for 10 min at 5% solvent B, followed by 17.5 min linear gradient to 15% solvent B. This intermediate mixture is then programmed isocratic for 13.5 min followed by a 1.0 min linear gradient to 50% solvent B. Acetic acid was added to lower pH of solvent to suppress ionization of carboxyl hydrogen of phenolic acids. Absorbance was measured at room temperature with a UV detector (254 nm) using C_{18} µ-Bondapak (31 x 0.8 cm) HPLC column with a flow rate of 1.0 ml/min. Sample extracts (5g in 20 ml 100% methanol) were prepared in methanol, vacuum dried, filtered through C_{18} Sap-Pak Cartridges and 0.45 µm pore size filters before injecting for analysis. Phenolic compounds (Sigma-Aldrich make) dissolved in spectral grade methanol were used as standards.

Observations and Results

The most significant changes in nodule metabolites during drought were level of sugars, proline and phenols. The role of sugars and proline has widely been acknowledged as osmo-protectants. Simultaneous appearance of phenols in the protective reactions during water stress prompted us to get a deep insight on the status of individual phenols. Separation was optimized using reverse phase chromatography (Waters, HPLC) with all the standards phenolic compounds of Sigma-Aldrich make (Figure 1).

The genotypes chosen for the present investigations revealed significant effects of cytokinin application under simulated water stress. Tannic acid, the first compound to be eluted in the profile was found to be more than double in quantity in genotype H208 (50.67 µg) than H96-99 (20.03 µg). The response of nodules was different in each genotype. Its contents were reduced by 71.7% (WS) and 63.7% (WS+Cyt) in H208 in comparison to controls. Reductions were slightly lesser in the presence of cytokinin (Table 1). A small quantity of gallic acid was detected in both the genotypes H208 (3.37 µg) than H96-99 (4.06 µg). Water deficit resulted in a sharp decline in its

levels by 43% in H208 (WS) in comparison to control. The presence of cytokinin was unable to prevent this loss as it did not elute after 5d of stress. Similarly, the disappearance in other genotype H96-99 was equally rapid in both treatments.

Quantity of gentisic acid was 4.57 µg in control nodules (H208) and not traceable in other H96-99. A significant and sharp increase was recorded at the end of 5d water stress in genotype H208. The percentage increase in both treatments was 62 and 100%. Interestingly, accumulation in genotype H96-99 was again very sharp wherein gentisic did not elute in the control nodulated plants. Under stress, its level was 7.56 µg and 7.19 µg both with and without BAP. *p*-Hydroxybenzoic acid (*p*-HBA) was measured as 10.42 µg and 5.24 µg in control genotypes H208 and H96-99. Its content showed marginal accumulations of 8.8% and 32.4% of control in H96-99 after 5d of stress. The response of H208 was different during stress both without cytokinin (1.5 fold sharp increase) and with cytokinin (contents rather reduced by 5.7% of control). Both the genotypes (H208-tolerant) and (H96-99 susceptible) differed in their response towards abiotic stress in accumulating *p*-HBA levels. Vanillic acid content of nodules increased 27% and 133% without BAP in control genotypes H208 and H96-99. This accumulation in presence of cytokinin was only 35% and 31%, respectively. The nodular response after 5d of stress was to immediately accumulate salicylic acid and pooled up levels were nearly 3.2 folds and 2.54 folds in H208 and H96-99 in comparison to control, respectively. This rise was checked to 2.48 and 2.22 folds, respectively in presence of cytokinin. Traces of *p*-coumaric acid were found in genotypes H208 and H96-99 and not traceable in H208 after 5d stress with BAP. A sharp accumulation (10 folds) noticed in H96-99 in presence of cytokinin.

Caffeic acid was not traceable under control and it accumulates under stress. A significant reduction in its levels was noticed with 6-BAP application. A major peak of cinnamic acid appeared in H208 and H96-99 control genotypes. The declining trend was noticed after 5d stress both with and without 6-BAP application (48.3% and 8.9%), respectively in genotype H208. Exogenous cytokinins induce cinnamic acid accumulations. The increase was 3.9% (without BAP) and 5.9% (with BAP) of control in H96-99 genotype. The estimated quantity of quercetin was more in control H208 (20.83 µg) than H96-99 (12.40 µg) genotypes. An apparent accumulations were noticed in genotype H96-99 both without (30.9%) and with 6-BAP (58.2%) in comparison to control.

Discussion

Characteristic identification of phenolics reveal that tannic acid, gallic acid, cinnamic acid, gentisic acid, *p*-hydroxybenzoic acid, salicylic acid and quercetin elute as major components and *p*-coumaric acid, vanillic acid and caffeic acid as minor components in the alcoholic extracts. Variation in the pooled-up phenolic components indicates water deficit stimulated expression of phenol-biosynthetic enzymes involved in Shikimic/Melonic acid pathways. Phenolic acids interaction with rhizobia led to physiological and biochemical changes resulting in their altered symbiotic ability

(Gamini, 2003). The first category of compounds like tannic acid, gallic acid, cinnamic acid and *p*-coumaric acid got reduced during stress and there was very little effect of cytokinin application except *p*-coumaric where its level recovered markedly in susceptible genotype H96-99.

Two major components salicylic and cinnamic acid behave oppositely under stress conditions. Role of phenolic compounds is both concentration and structure based as the possible agents for the legume-rhizobial symbiosis. *p*-Coumaric acid and *trans*-cinnamic acid were reported to reverse the ABA induced stomatal closure (Laloraya *et al.*, 1986). Salicylic acid (SA), a possible signal molecule participates in hypersensitivity reaction of cells and the formation of Systemic Acquired Resistance (SAR) of plant (Vasyukova *et al*., 1999; Klessig *et al*., 2000; Molodchenkova, 2001). Link between SA accumulation and increased H_2O_2 was based upon inhibition of enzymes catalase and peroxidase (Rao *et al*., 1997). H_2O_2 induces benzoic acid which is a precursor of SA and rhizobia produce unidentified elicitor inducing SA accumulation by initiating start up of protective reactions (Leon *et al*., 1995; Schulze and Kondrosi, 1998). Exogenous application of SA reported to inhibit rhizobia penetration in root tissues contributing to increased endogenous SA and H_2O_2 content of the nodules (Glyanko *et al*., 2003) and alleviate adverse effects of drought stress by inducing endogenous plant hormones like IAA, GA3 and CK (War *et al*., 2011; Sadeghipour and Aghaei, 2012). Cytokinins are formed by bacterial symbionts that colonize plant tissues and not by the plants at all (Holland, 1997). Thus, cytokinins plays an important role in nodular metabolism by antagonizing the deleterious effects of water stress. Phenolic compounds not only determine tolerance level of a genotype but, also interact with cytokinins in regulating defense mechanism of the root nodules. These compounds especially Salicylic acid participates in initiation of protective reactions of the host plant, *p*-Hydroxybenzoic acid and *p*-Coumaric acid determine the tolerance and susceptibility levels of a genotype, respectively.

References

[1] Catsky, J., Pospinsilova, J., Kaminek, M., Gaudinova, A., Pulkrabek, J. and Zahradnicek, J. (1996). Seasonal changes in sugar beet photosynthesis as affected by exogenous cytokinin N^6-(m-hydroxybenzyl)-adenosine. *Biol. Plant.*, 38: 511-518.

[2] Davies, P.J. (1995). The plant hormone concept: concentration, sensitivity and transport. In: Davies, P.J. (ed.), *Plant Hormone*, 13-38.

[3] Gamini, S. 2003. Phenolic acids: Possible agents of modifying N_2-fixing symbiosis through rhizobial alterations? *Plant and Soil*, 252(2): 385-395.

[4] Glyanko, A.K., Makarova, L.E., Luzova, G.B., Mironova, N.V. and Vasilieva, G.G. 2003. Impact of salicylic acid on symbiotic relations between peas and *Rhizobium leguminosarum*. *Acad. Jour.*, 10: 1-8.

[5] Hahn, D.H., Faubion, J.M. and Rooney, L.W. (1983). Sorghum phenolic acids, their high performance liquid chromatography separation and their relation to fungal resistance. *Cereal Chem.*, 60: 255-259.

[6] Holland, M.A. (1997). Occam's razor applied to hormonology. Are cytokinins produced by plants? *Plant Physiol.*, 115: 865-868.

[7] Kawano, T. (2003). Role of reactive oxygen species- generating peroxidase reactions in plant defense and growth induction. *Plant Cell Rep.*, 21: 829-837.

[8] Klessig, D.F., Durner, J. and Nood, R. (2000). Nitric oxide and salicylic acid signaling in plant defense. *Proc. Natl. Acad. Sci. (USA)*, 97(16): 8849-8855.

[9] Laloraya, M.M., Nozzolillo, C, Purohit, S. and Stevenson, L. 1986. Reversal of abscisic acid-induced stomatal closure by *trans*-cinnamic and *p*-coumaric acid. *Plant Physiol.*, 81(1): 253-258.

[10] Leon, J., Lawton, M.A., Raskin, I. (1995). Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiol.*, 108: 1673-1678.

[11] Maffei, M.E., Mithofer, A. and Boland, W. (2007). Insects feeding on plants: Rapid signals and responses preceding the induction of phytochemical release. *Phytochem.*, 68: 2946-2959.

[12] Molodchenkova, O. 2001. Cultural plants. *Physiol. Biochem.*, 33: 463-473.

[13] Naqvi, S.M. (1995). Plant/crop hormones under stressful conditions. In: Pessarakli, M. (ed.), *Handbook of Plant and Crop Physiology*: 645-660. Marcel Dekkar, New York-Basel-Hong Kong.

[14] Rao, M.V., Paliyath, G., Ormord, D.P., Murr, D.P. and Watkins, C.B. 1997. Influence of salicylic acid on H_2O_2 production, oxidative stress and H_2O_2 – metabolizing enzymes (salicylic acid mediated oxidative damage requires H_2O_2). *Plant Physiol.*, 115(1) 137-149.

[15] Sadeghipour, O. and Aghaei, P. 2012. The role of exogenous salicylic acid (SA) on phytohormonal changes and drought tolerance in common bean (*Phaseolus vulgaris* L.). *Jour. Biodiver. Environ. Sci.*, 2(12): 8-15.

[16] Schulze, M. and Kondrosi, J. (1998). Regulation of symbiotic root nodules development. *Ann. Rev. Genet.*, 32: 33-57.

[17] Vasyukova, N.I., Gerasimova, N.G. and Ozeretskovskaya, O.L. 1999. The role of salicylic acid in plant resistance to diseases (Review). *Appl. Biochem. Microbiol.*, 35: 557-563.

[18] Vicent, M.R.S. and Plasencia, J. (2011). Salicylic acid beyond defense: its role in plant growth and development. *Jour. Experim. Bot.*, 62: 3321-3338.

[19] War, A.R., Paulraj, M.G., War, M.Y. and Ignacimuthu, S. (2011). Role of salicylic acid in induction of plant defense system in chickpea (*Cicer arietinum* L.). *Plant Signal. Behav.*, 6(11): 1787-1792.

PLATE 1

Figure 1: A standard profile (X-axis: retention time (min.) and Y-axis: absorbance units) of ten phenolic acids namely tannic acid, gallic acid, gentisic acid, p-hydroxybenzoic acid, vanillic acid, salicylic acid, p-coumaric acid, caffeic acid, cinnamic acid and quercetin in the order of their elution with reverse phase chromatography (Waters, HPLC).

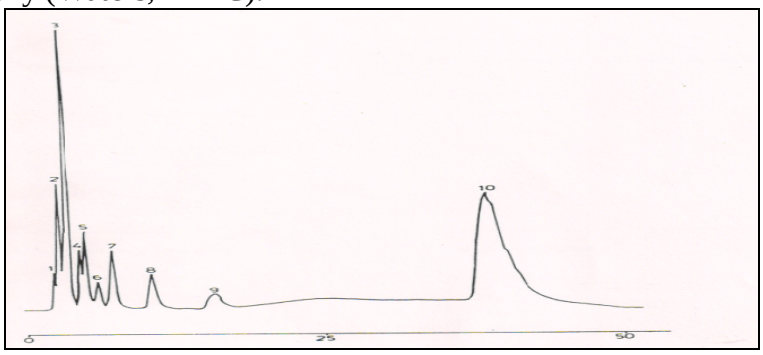

Table 1: Nodular content of individual phenolic compounds after 5d of water stress in chickpea genotypes.

No.	Phenolic Compound	Genotype H208 µg/g fr wt			Genotype H96-99 µg/g fr wt		
		Control	WS	WS+Cyt	Control	WS	WS+Cyt
1	Tannic acid	50.67^{a} ±1.24	14.34^{b} ±0.96	18.39^{c} ±0.86	20.03^{a} ±1.38	20.06^{b} ±0.79	21.63^{c} ±1.02
2	Gallic acid	3.37^{a} ±0.76	1.92^{b} ±0.24	--	4.06^{a} ±0.84	--	--
3	Gentisic acid	4.57^{a} ±0.12	7.40^{b} ±0.24	9.14^{c} ±0.87	--	7.56^{b} ±0.24	7.19^{c} ±0.86
4	p-hydroxy benzoic acid	10.42^{a} ±1.72	15.63^{b} ±0.64	4.82^{c} ±0.12	5.24^{a} ±0.14	5.70^{b} ±0.22	6.94^{c} ±0.31
5	Vanillic acid	2.46^{a} ±0.04	3.12^{b} ±0.03	3.32^{c} ±0.25	1.86^{a} ±0.02	4.33^{b} ±0.44	2.43^{c} ±0.16
6	Salicylic acid	50.42^{a} ±3.43	161.34^{b} ±10.14	125.04^{c} ±8.65	60.28^{a} ±3.98	153.11^{b} ±6.56	133.82^{c} ±9.24
7	p-Coumaric acid	1.56^{a} ±0.02	0.008^{b} ±.001	--	0.08^{a} ±0.005	--	0.82^{c} ±0.05
8	Caffeic acid	--	0.38^{b} ±0.001	--	--	0.32^{b} ±0.003	0.09^{c} ±0.004
9	Cinnamic acid	111.96^{a} ±10.47	101.99^{b} ±8.97	57.88^{c} ±4.35	34.11^{a} ±3.24	32.98^{b} ±2.12	36.12^{c} ±3.23
10	Quercetin	20.83^{a} ±2.45	20.12^{b} ±1.97	22.34^{c} ±0.86	12.40^{a} ±2.32	16.23^{b} ±1.80	19.62^{c} ±0.95

Note: Values are mean± standard error, n=3. Different superscript letters on mean values along the rows indicate significant differences within P≤0.05 according to Tukey's HSD range test.