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Original article

Studies on antioxidants and peroxidase isoenzymes in seedlings of twelve cultivars with four different durations of flowering time in pigeon pea (*Cajanus cajan* (L.) Millspaugh)

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ABSTRACT

Objective: The present study is an attempt to understand the role of enzymatic and non-enzymatic antioxidants as well as peroxidase isoenzymes in order to differentiate the four flowering durations of pigeon pea cultivars.
Methods: The assay of superoxide dismutase, catalase, peroxidase, ascorbate oxidase, ascorbic acid, reduced glutathione, lipid peroxidation, protein and phenolic content was done for the evaluation of antioxidant activity. For peroxidase isozymes assay, native-PAGE was carried out with 10% acrylamide gels at 4 °C without SDS and β-mercaptoethanol.
Results: Among all the cultivars ICP 15599, which is an extra-early flowering variety showed highest levels of both enzymatic and non-enzymatic antioxidants and least lipid peroxidation. Isoenzyme marker analysis of POD showed six common isoforms in all the 12 varieties and an additional isoform in ICP 15599.
Conclusion: Among four different flowering durations extra-early group showed highest activity of enzymatic and non-enzymatic antioxidants, whereas lipid peroxidation was least. The peroxidase isozyme analysis showed there is no specific isoform for flowering time identification, but however there is one marker for extra-early cultivar (ICP 15599).

1. Introduction

Germination and flowering are both fundamental developmental transitions that require precise environmental sensing and responses to multiple seasonal cues. The combination of these two phenological traits determines the overall life cycle and generation time of many plants.[1] Because both transitions respond to similar seasonal cues, it is logical to hypothesize that genetic pathways of these two life-history transitions share common elements. Pigeon pea (*Cajanus cajan* (L.) Millsp., Family – Fabaceae), is one of the major pulse crops of the tropics and sub-tropics and is mostly intercropped with cereals. It is a short-day plant,[2] and so the onset of flowering is delayed in longer rather than shorter days.[3] Based on time of flowering, pigeon pea genotypes have been classified into four major maturity groups: the extra-early, early, medium and late genotypes.

Seed germination and flowering are complex processes requiring a multidisciplinary approach in analysis.[4] The sequence of the metabolic pattern that occurs during flowering involves the activation of specific enzymes at the appropriate times and regulation of their activity. Accumulation of active oxygen species (AOS), during seed imbibition, leads to germination.[5] Appearance of AOS in the plant cells is generally linked with the involvement of free radical in plant development, as well as its interaction with the environment.[6,7] On the other hand, some of them, such as H_2O_2 and O_2^- are proposed to have a signaling role in the cell during stress action.[8,9] A regulated balance between oxygen radical production and destruction is required if metabolic efficiency and function are to be maintained in both optimal and stress conditions. In plant cells the detoxifying enzymes (peroxidase, catalase, superoxide

Abbreviations: AOS, active oxygen species; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; ASO, ascorbate oxidase; ASH, ascorbic acid; GSH, reduced glutathione; MDA, malondialdehyde; PAGE, polyacrylamide gel-electrophoresis; SDS, sodium dodecyl sulfate.

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dismutase and ascorbate oxidase) along with reduced glutathione and ascorbic acid have an important role throughout plant ontogeny, from seed germination[5] to growth and development.[10,11] Malondialdehyde (MDA) is considered sensitive marker commonly used for assessing membrane lipid peroxidation.[12]

The increased economic importance of the crop has stimulated the development of new cultivars. Therefore, identification of varieties became very important in seed certification and crop breeding programs to screen different plant genotypes. At present, morphological features are commonly used to identify crop cultivars. For some plant species, identification based on plant and seed morphology has been unreliable, because morphological characters can be affected by environmental conditions.[13] Moreover, a cultivar also must be judged by an individual who possesses a thorough knowledge of the cultivar at the precise time. For morphological characterization, the plant must be grown to flowering or fruiting stage, which is space and time consuming. Therefore, it is desirable if a cultivar identification system could be developed, based on biochemical techniques.[14]

Hence the present study is an attempt to understand the role of enzymatic and non-enzymatic antioxidants as well as peroxidase isoenzymes in order to differentiate the four flowering durations of pigeon pea.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 2,6-dichloro phenol indophenol, reduced glutathione (GSH), thiobarbituric acid (TBA), acrylamide, bis acrylamide, sodium dodecyl sulfate (SDS), ammonium persulphate (APS), β-mercaptoethanol, coomassie brilliant blue R 250 were obtained from Himedia Laboratories Pvt. Limited, Mumbai, India. Gallic acid, mercuric chloride, hydroxylamine hydrochloride, sodium carbonate, potassium permanganate, oxalic acid, meta phosphoric acid, 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Qualigens, Mumbai, India. Nitroblue tetrazolium (NBT) were obtained from Merck Limited, Mumbai, India.

2.2. Plant material

Twelve pigeon pea cultivars belonging to four flowering durations namely extra-early (ICP 7220, ICP 15598, ICP 15599), early (ICP 26, ICP 28, ICP 1124), medium (ICP 472, ICP 3437, ICP 7118), late (ICP 1342, ICP 1406, ICP 1433) were used in the present study. The seeds were obtained from ICRISAT, Patancheru, Andhra Pradesh, India. Seeds of uniform size were selected and soaked in distilled water for 2 h and were surface sterilized with 0.1% mercuric chloride for 2 min. The seeds were then washed thoroughly with sterile distilled water. The washed seeds were then spread over petri dishes lined with two-layered moistened filter paper. The seeds of these cultivars were allowed to germinate at 30 ± 2 °C for 6 days under a photoperiod of 16 h day light and 8 h dark. Then the 6 days old seedlings were collected and used for experimental analysis. Radicle emergence of 2 mm was considered as germination.[15] Germination percentage was determined for each cultivar. The experiment was repeated thrice with sample size of 20 seeds.

2.3. Preparation of extract

About 1 g seedlings of each cultivar were homogenized separately with 7.5 mL of pre-chilled phosphate buffer (0.1 M, pH 7.6), containing 0.1 mM EDTA in chilled mortar and pestle. The homogenate was squeezed through double layered cheese cloth and centrifuged (Sorvall Instrument RC5C, Rotor SS-34) at 16,000 rpm for 15 min at 4 °C. The supernatant was used for the assay of SOD, CAT, POD, and ASO also for non-enzymatic antioxidants like total phenols, reduced glutathione, ascorbic acid, and proteins. The assay of SOD was carried by the method of Beauchamp and Fridovich based on the reduction of Nitroblue tetrazolium (NBT).[16] CAT activity was assayed by the titrimetric method described by Radhakissnan and Sarma.[17] Peroxidase (POD) activity was assayed spectrophotometrically (Shimadzu UV-265, UV–visible recording spectrophotometer) with O-dianisidine as hydrogen donor.[18,19] Assay of ASO activity was carried out according to the procedure of Vines and Oberbacher.[20] The determination of ASH was carried out by the procedure given by Sadasivam and Balasubramanian.[21] The GSH content was determined by the Boyne and Ellman method.[22] Lipid peroxidation (LPO) of the plant extract was determined by estimating the MDA content following the method of Heath and Packer with slight modification.[23] Total protein was estimated by the method of Lowry et al[24] with Bovine Serum Albumin as standard. The total phenolic content was determined spectrophotometrically by the method described by Sadasivam and Manickam.[19] For POD isozymes assay, native-PAGE was carried out by a modified method of Davis[25] with a 10% acrylamide gels at 4 °C without SDS and β-mercaptoethanol.

2.4. Statistical analysis

Each experiment was repeated three times. Analysis of variance was conducted using one-way ANOVA test using SPSS 9.01 for Microsoft Windows and mean separations were carried out using Duncan's Multiple Range Test (DMRT). Statistical significance was determined at 5% ($P < 0.05$) level.

3. Results

3.1. Germination

Germination percentage of the 12 cultivars (four different durations of flowering time) of pigeon pea was shown in Table 1. All cultivars showed germination percentage of above 80% except ICP 15598 and ICP 15599, which showed 78.33% and 65% respectively [Table 1].

3.2. Enzymatic antioxidants

Duncan grouping showed that there is significant difference between four different groups in POD, CAT and ASO activities,

Table 1
Germination percentage of 12 pigeon pea cultivars.

Duration	Type of cultivar	Germination percentage (%)[C]
Extra-early	ICP 7220	90.0 ± 5.0
	ICP 15598	78.33 ± 5.77
	ICP 15599	65.0 ± 5.0
Early	ICP 26	90.0 ± 0.0
	ICP 28	100.0 ± 0.0
	ICP 1124	100.0 ± 0.0
Medium	ICP 472	95.0 ± 5.0
	ICP 3437	100.0 ± 0.0
	ICP 7118	91.67 ± 2.89
Late	ICP 1342	95.0 ± 5.0
	ICP 1406	76.67 ± 2.89
	ICP 1433	86.67 ± 2.89
		$F = 26.69$[B]

Values represent the mean ± standard deviation of three independent experiments.
[B] Not significant at 5% level.
[C] The values represent the mean ± standard deviation of three independent experiments.