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Profile of Antioxidants and Scavenger Enzymes during Different Developmental Stages in *Vigna radiata* (L.) Wilczek (Mungbean) under Natural Environmental Conditions

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Abstract Leaf samples of *Vigna radiata* (L.) Wilczek (Mungbean) were collected at different developmental stages *viz*. 6, 25, 45, 60 and 75 days, grown under natural environmental conditions and were analyzed for studying the profile of antioxidants – carotene, xanthophyll and ascorbic acid along with scavenger enzymes – catalase, peroxidase and ascorbic acid oxidase. This paper discusses the changes in the enzymatic and non-enzymatic antioxidant content in correlation to the physiological and metabolic status of the crop in different growth stages. Our work indicates that 25 to 45 days after sowing (DAS) is the favorable period (active growth) and 60 DAS is the stressful stage (comparable to senescence) in the *Vigna radiata* life cycle in terms of antioxidant profile and scavenging enzymes. The findings will address future problems related to stress physiology and senescence of this particular legume. It may lead to further studies for delaying senescence of this particular crop using Recombinant DNA technology.

Keywords Antioxidant, Scavenging enzymes, Mungbean, Carotene, Xanthophyll, Ascorbic acid, Catalase, Peroxidase, Ascorbic acid oxidase

1. Introduction

Plants possess enzymatic and non-enzymatic antioxidative defense systems in the organelles of their cells. Catalase, peroxidase, and Superoxide dismutase, enzymes in the ascorbate-glutathione cycle, such as ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase are examples of major antioxidative enzymes [1-2]. In this study out of these, three marker enzymes catalase, peroxidase and ascorbic acid oxidase were selected as indices of the physiological and metabolic status of different developmental stages in legume *Vigna radiata* (L.) Wilczek (Mungbean) under natural environmental conditions. The major non-enzymatic antioxidants are ascorbate (vitamin C), carotenoid (carotene and xanthophylls), glutathione, α-tocopherol (vitamin E), β-carotene, and flavonoids; these antioxidants are distributed chiefly in chloroplasts, but also occur in other cellular compartments, such as mitochondria and peroxisomes [1]. Of these, two non-enzymatic antioxidants *viz*. carotenoid (carotene and xanthophylls) and ascorbic acid were selected as work parameters.

Mungbean is an important traditional leguminous crop of India characterized by a relatively high content of protein and is a short summer season crop. Abundant information is available on legume plant responses under different kinds of imposed abiotic stresses [3-4]. Information is relatively sparse on activities of scavenging enzymes and antioxidants at the various developmental stages in legumes under natural environmental conditions, which is why this particular research work was specifically carried out. This research was undertaken to study the response of the mungbean plant under natural tropical conditions of Kolkata, India in different developmental stages. The antioxidant and enzyme profile in the different stages could serve as an indicator of the physiological status of the plant under natural environmental conditions which in turn could indicate the most optimum and/or stressful developmental stage for further studies in future. Since many different agriculturally important traits are affected by senescence, which in turn, is strongly affected by the scavenging enzymes and antioxidants under study, this work related to antioxidant and

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scavenger enzyme profile in different developmental stages of mungbean (an important crop plant) may contribute to address this particular aspect.

2. Materials & Methods

Vigna radiata (L.) Wilczek (Mungbean), chosen for the experimental work is an excellent and cheap source of high quality protein. Mungbean is consumed as whole grain as well as pulse in a variety of ways. It is also a green manuring crop. Being a leguminous crop, it has the capacity to fix atmospheric nitrogen. The soil was prepared by mixing farmyard manure in accordance with normal agronomic practices to avoid any changes in edaphic conditions. The clay pots of 14 inch diameter were filled with 8 kg of soil per pot. The sieve garden soil and sand were mixed in the ratio of 3:1. After preparation of pots, they were arranged in rows in the experimental garden at Govt. College of Leather Technology, Salt Lake, Kolkata (22.34^0 North and 88.24^0 East), West Bengal, India. The experiments were conducted in the summer months of March-June 2013. The Meterological Data were collected from Regional Meterological Centre, Alipore, Kolkata. (Table 1).

Certified seeds of *Vigna radiata* (L.) Wilczek variety SML668 was obtained from National Seeds Corporation Ltd., Sector IV & V, Block AQ, Kolkata. Healthy surface sterilized [5] seeds of mungbean were selected and soaked in distilled water for 6 hours to initiate better germination and for early seedling development. These pots were placed in the open air of the wire netting enclosure to save seeds and young seedlings from birds. After complete germination, number of seedlings per pot was reduced to 4 by thinning to ensure that 4 plants were of more or less equal size and vigour. All pots were watered uniformly throughout the experimental period in order to maintain constant soil moisture. Healthy leaf samples were collected at 6, 25, 45, 60 and 75 days after sowing (DAS) and cleaned with sterile distilled water before weighing. All leaf samples were homogenized in mortar and pestle with respective buffer for estimation of different enzymatic and non enzymatic antioxidants.

Non-enzymatic antioxidants *viz.* Carotene and xanthophyll (carotenoid) were estimated according to the method of Davies [6] and Ascorbic acid content according to Mitsui and Ohta [7]. Ascorbic acid oxidase activity was assayed according to Oberbacher and Vines [8]; scavenging enzymes *viz.* Catalase enzyme was assayed according to method of Gasper and Lacoppe [9] and Peroxidase according to Chance and Maehly [10] with slight modifications. Protein content was estimated according to Lowry [11]. The data obtained from three replications were statistically analyzed. Standard Deviation (SD) and Critical Difference (CD) values of different developmental stages at 5% were calculated from the analysis of variance (ANOVA) table. SD of the data is shown in the graphs and the calculated CD value is also provided.

3. Results and Discussion

Meterological data spanning the months of March-June (summer season) 2013 were collected from the Regional Meterological Centre, Alipore, Kolkata. Monthly mean values of temperature, photoperiod, light intensity, relative humidity and rainfall were noted, from which the summer season mean values were calculated for the time period of the experimental period (Table 1).

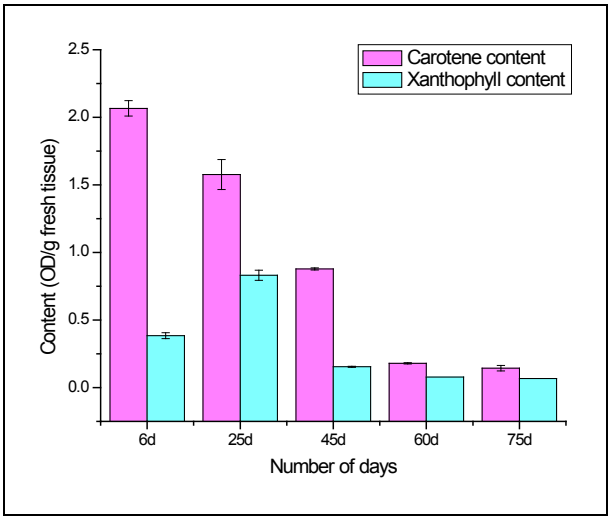
Table 1. Meteorological Data of Summer Season (March-June 2013) showing Mean Values

Temperature (^{o}C)	36.5
Sunshine Hours (Hour)	13.5
Light Intensity (Lux)	68,000
Relative Humidity (%)	79
Rainfall (mm)	117

Carotenoids are lipophilic organic compounds located in the plastids of both photosynthetic and non-photosynthetic plant tissues. Carotene pigments such as beta-carotene or xanthophylls like lutein and zeaxanthin are widely distributed in nature and serve as antioxidants [12]. In terms of their antioxidant properties, carotenoids can protect photosystems in one of four ways: (1) by reacting with Lipid Peroxidation products to terminate chain reactions, (2) by scavenging $^1O_2$ and dissipating the energy as heat, (3) by reacting with 3Chl* or excited chlorophyll (Chl*) molecules to prevent the formation of $^1O_2$, or (4) by dissipating excess excitation energy through the xanthophyll cycle. The main protective role of β-carotene in photosynthetic tissue may be accomplished via direct quenching of 3Chl*, which prevents $^1O_2$ generation and thereby inhibits oxidative damage [13]. During quenching of 3Chl*, energy is transferred from Chlorophyll to carotenoid, which subsequently dissipates the energy in a non-radiative form (i.e., heat). Thus, carotenoids act as a competitive inhibitor of $^1O_2$ formation and this is aided by their proximity to Chl in the light-harvesting complex. Zeaxanthin appears to facilitate the conversion of 3Chl* to 1Chl* more efficiently than does β-carotene [14].

Carotenoid (carotene and xanthophyll) content was found to decline significantly during the entire developmental phase *i.e.* 6>25>45>60>75 DAS (Fig.1). A high carotenoid content in the earlier developmental stages *viz*. 6, 25 and 45 DAS indicate efficient scavenging and detoxification of harmful reactive oxygen species. Thus the relatively high carotenoid content in the vegetative growth period implies optimum physiological status of the plant as compared to the 60 and 75 DAS stages where the sharp decline in carotenoid content is indicative of the approaching/ongoing senescence in the plant. In earlier works on crop plants, carotenoid content was reported to decline significantly from the pre-flowering to the post-flowering stage under natural environmental conditions and the decreased carotenoid content can be a significant reason ushering senescence [15-18]. There have been earlier reports showing the decrease in carotenoid content during periods of

environmental stress [19-20]. In this study the decline in carotenoid content in 60 and 75 DAS stages is indicative of a stressful phase which is not imposed but is a part of the plant life cycle *i.e.* senescence.

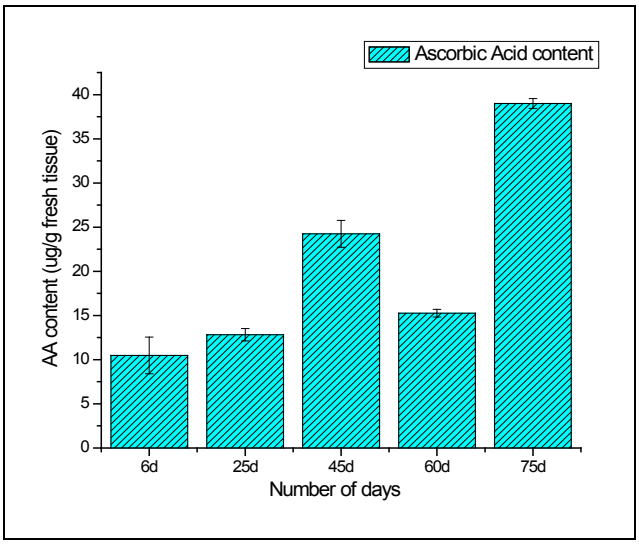


CD value of developmental stage (5% level) for Carotene= 1.2374
CD value of developmental stage (5% level) for Xanthophyll = 0.4690

Figure 1. Carotene & Xanthophyll content

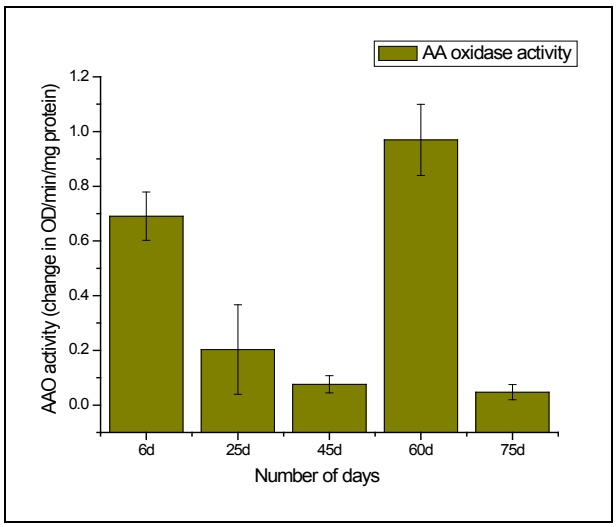
Ascorbic acid content increases from 6<25<45 DAS. There is a sharp drop at 60 DAS and then a rise at 75 DAS (Fig.2). Total carotenes and ascorbic acid are important quality attributes for nutritional purposes and a high ascorbic acid content is generally considered a good index to evaluate the freshness of the product. In previous works, a high ascorbate content was associated with a favourable growing period of crop plants under natural environmental conditions, while diminished ascorbate was indicative of poor physiological status and impaired metabolism [17-18]. Seasonal environmental stress was thus significantly marked by decline in antioxidant (carotenoid and ascorbate) content [15-18].

Ascorbate (Vitamin C) acts as a potent and probably the most important hydrophilic antioxidant and is a good reducing agent and scavenger of hydroxyl and oxygen free radicals [21-22]. Ascorbic acid is one of the most powerful antioxidants and is present in most plant cell types, organelles, and the apoplast [23-24]. Under physiological conditions, ascorbic acid exists mostly in the reduced form (90% of the ascorbate pool) in chloroplasts [24]. The ability of ascorbic acid to donate electrons in a wide range of enzymatic and non-enzymatic reactions makes ascorbic acid the main ROS-detoxifying compound in the aqueous phase. Ascorbic acid can directly scavenge $O_2^{\cdot -}$, $OH^{\cdot}$, and $^1O_2$. The steady increase in ascorbate content from 6-45 DAS indicates that there is efficient detoxification of free radicals during the vegetative growth period. At 60 DAS, the drop in ascorbate content is indicative of a stressful period implying reduced efficiency in ROS scavenging possibly aiding senescence. At 75 DAS the marked increase in ascorbate is possibly due to the greatly diminished activity of ascorbic acid oxidase (Fig.3).



CD value of developmental stage (5% level) for A Acid = 17.026

Figure 2. Ascorbic Acid Content



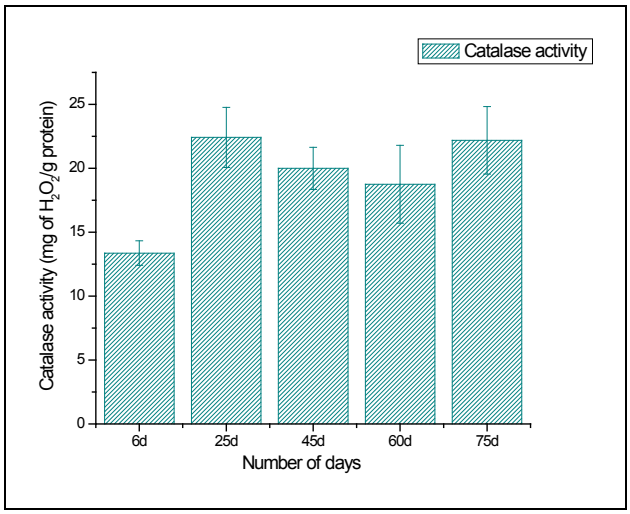
CD value of developmental stage (5% level) for AA Oxidase = 0.6099

Figure 3. Ascorbic Acid Oxidase Activity

Ascorbic acid oxidase (EC 1.10.3.3) is a multi-copper enzyme that catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. In this work the enzyme activity declines from 6>25>45 DAS. There is a considerable rise at 60 DAS followed by significant drop at 75 DAS (Fig. 3). From this data it is evident that ascorbic acid and ascorbic acid oxidase are inversely related to one another i.e. a high ascorbic acid content is associated with low ascorbic acid oxidase activity (45 & 75 DAS) and vice versa.

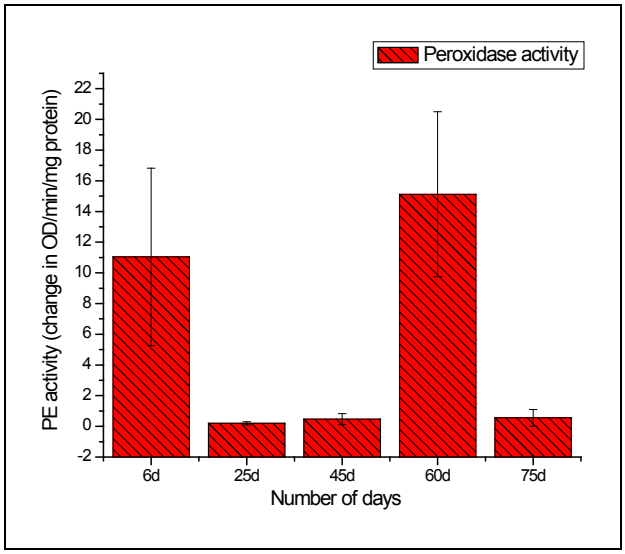
Catalase (EC 1.11.1.6) increases from 6 to 25 days and decreases from 25 to 45 and 60 DAS. The activity rises from 60 to 75 DAS (Fig.4). Catalase (H_2O_2 oxidoreductase) is a heme-containing enzyme that catalyses the dismutation of H_2O_2 into H_2O and O_2. The enzyme occurs in all aerobic eukaryotes and its function is to remove the H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty

acids, photorespiration, purine catabolism and during oxidative stress [25-26]. Moreover, there is proliferation of peroxisomes during stresses, which might help in the scavenging of H_2O_2 that diffuses from the cytosol [27]. The increase in catalase activity from 6 to 25 DAS indicates that the enzyme effectively scavenges the harmful radicals to maintain an optimum state so that the plant can continue its vegetative growth and move onto the reproductive stage. The diminished activity of catalase from 25 to 45 and 60 DAS implies that the plant is in an optimum state and physiological status. The rise in catalase activity from 60-75 DAS is indicative of the approaching senescence which acts as a natural stress. ROS are known to increase under stress [28-29]) and the increased enzyme activity is an attempt to combat the natural stress *i.e.* senescence.



CD value of developmental stage (5% level) for Catalase = 6.0082

Figure 4. Catalase Activity



CD value of developmental stage (at 5% level) for Peroxidase = 11.3092

Figure 5. Peroxidase Activity

Peroxidase (EC 1.11.1.7) scavenges H_2O_2 by catalyzing the divalent reduction of H_2O_2 to water using a variety of reductants. Peroxidase is a stress marker enzyme known to be very active under stressful conditions and is also reportedly very active during senescence [30]. In this work the enzyme activity declines sharply from 6 to 25 DAS, followed by a slight increase at 45 DAS. Activity then rises sharply from 45 to 60 DAS and declines significantly at 75 DAS (Fig.5). Peroxidase activity is possibly high at 6 DAS to maintain an optimum metabolic status by efficient scavenging of free radicals. The very low peroxidase enzyme activity at 25 and 45 DAS indicates that conditions are optimum for growth and metabolism. The manifold rise in enzyme activity at 60 DAS implies the prevalence of stressful conditions possibly as senescence. Abundant production of free radicals during senescence must have stimulated high enzyme activity which makes an effort to scavenge the ROS. The drastic decline of enzyme activity at 75 DAS is possibly indicative of the inability of the enzyme to scavenge the free radicals and combat senescence, a stage when free radicals have been reported to be produced in abundance [31].

4. Conclusions

From this work, it indicates 60 DAS to be the point in the plant life cycle when the plant shifts from an optimum phase (active growth) to a stressful condition (comparable to post flowering/senescence). This is amply justified by the low ascorbic acid content accompanied by low carotenoid content i.e. both kinds of non-enzymatic antioxidants are significantly low as compared to the previous favorable stage of 45 DAS. Both the scavenging enzymes viz. catalase and peroxidase show elevated activities at this particular stage i.e. 60 DAS in order to detoxify the free radicals and combat the existing natural stress of senescence. Ascorbic acid oxidase activity is also high which is the cause of low ascorbic acid content. From this work, it is also evident that the period from 25-45 DAS is the most favorable in terms of antioxidant profile and scavenging enzymes. Thus the physiological status is optimally maintained during this phase resulting in active growth and flowering. As our work indicates the favorable period (25-45 DAS) as well as the stressful (60 DAS) stage (post flowering leading to senescence) in the *Vigna radiata* life cycle in terms of antioxidant profile and scavenging enzymes, these findings may contribute in the future research about stress physiology and senescence of this particular legume.

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