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NUCLEAR PLOIDY LEVEL VARIATION IN ANTIOXIDANT POTENTIAL OF MULTIPURPOSE LEGUME LATHYRUS SATIVUS L. UNDER COPPER STRESS

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ABSTRACT

Antioxidant defense capabilities were investigated in three ploidy levels of grass pea (Lathyrus sativus L.) genotypes under un-treated and treated (40 mg/l CuCl2) conditions. Significant differences were observed between ploidy levels and even within same ploidy level. Usually, tetraploids exhibited highest capability of enzyme activities, followed by triploids and diploids. Upon exposure to Cu, enzyme activities and reduced ascorbate content decreased significantly in diploids and triploids (triplo II-obtained from diploid × type II auto-tetraploids), hampering in scavenging of reactive oxygen species and consequently, resulting in high magnitude of lipid peroxidation. Decreased ascorbate level in their leaves might be related to below normal activity of dehydroascorbate reductase which also affected normal functioning of ascorbate peroxidase under Cu exposure. By contrast, triplo I (obtained from diploid × type I auto-tetraploids) and both types of tetraploids showed normal to enhanced capacities of antioxidant defense activities under Cu treatment. Higher ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase activities in Cu- treated triplo I and both tetraploid genotypes suggested efficient scavenging of reactive oxygen species and regenerations of reduced forms of ascorbate and glutathione in these three genotypes. Absence of any type of oxidative damage in triplo I and both types of tetraploids was evident from quite normal level of malondealdehyde, a cytotoxic aldehyde from membrane lipid peroxidation, content in their leaves. The results suggested far greater tolerance of tetraploids over diploids, while two types of triploids exhibited differential response to Cu treatment.

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KEY WORDS

Antioxidant defense; Ascorbate; Cu-stress; Glutathione; Lathyrus sativus L.; Nuclear ploidy

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[I] INTRODUCTION

Lathyrus sativus L. or grass pea, belonging to the family Fabaceae and sub-family Papilionoideae under the tribe Vicieae, is a winter cool-season legume crop grown worldwide mainly as food (pulse) and fodder in Indian Sub continent, Africa, Australia, South America, and in the Mediterranean [1]. The crop is remarkably hardy to diverse types of climatic conditions (cold, high temperature, water-logging etc.), biotic, and abiotic stresses including water stress, drought, salinity, and heavy metals [2], and also is a novel plant in different types of biological research such as legume cytogenetics, genomics and proteomics, stress biology and mutation genetics [3, 4]. With 2n=2x=14 chromosomes, the crop is strictly diploid across the genotypes, but exhibits karyomorphological variation in nuclear chromosomal morphology [5]. Like other legumes, grass pea seeds are highly rich in natural antioxidants, flavonoids and polyphenols [6], the variation of which in many plant species depends on genomic ploidy level and its stability across generations [7].

The antioxidant potential of a plant species comprises of antioxidant composition and activity which can further be divided into enzymatic and non-enzymatic defense. The formation of free radical is a consequence of aerobic life cycle and is usually under tight regulation [8]. Among the non-enzymatic antioxidant, ascorbate (AsA), glutathione (GSH), different flavonoids and polyphenolic content play pivotal role in scavenging of reactive oxygen species (ROS). On the other hand, enzymatic metabolism of ROS predominantly involves superoxide dismutase (SOD), ascorbate peroxidase (APX), catalases (CAT) and glutathione reductase (GR). While the former three is involved in metabolism of hydrogen peroxide (H2O2), the latter maintains redox state of both glutathione and ascorbate in normal level [8]. Accumulating evidences indicate that increasing chromosomal ploidy level may enhance the antioxidant potential of crop plants to a considerable extent [9], which can be exploited in the benefit of formulations of functional foods against human diseases [7]. In grass pea and related legumes like beans and lentils, strong evidences for alteration in antioxidant defense response have been revealed in genotypes exhibiting alterations in antioxidant defense [10-12]. In ploidy level, higher than diploids significant variation in antioxidant response to induced salinity stress was observed in primary trisomic, tetrasomics and double-trisomic lines [9] with severe dosage imbalance, isolated and characterized in grass pea [3, 4]. Although Cu is an essential micronutrient for plant growth, its high bioavailability in soils may be toxic to plants, causing inhibition of growth and oxidative stress [13]. It is known to damage integrity of cell membrane by binding to

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sulphydryl groups of membrane proteins and inducing lipid peroxidation through the generation of reactive oxygen species [13]. Evidences from several plant species reveal that Cu causes oxidative stress by modulating the activities of antioxidant enzymes [13], particularly expressions of SOD, APX, DHAR, GR and CAT in plant. Despite immense importance of polyploidy with more dosage in balanced genomes towards improvement of antioxidant potential and tolerance against abiotic stress, no such studies have been carried out in antioxidant rich grass pea. Hence, the present study was carried out with an objective to analyze the antioxidant properties of raw seeds samples of different autotetraploids, triploids and their progenies including diploid line in grass pea. The analysis included analysis of total ascorbate and assessment of antioxidant enzyme activities and levels of lipid peroxidation in grass pea leaves at different ploidy levels. Activities of five prominent ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and catalase (CAT) will be performed.

[II] MATERIALS AND METHODS

2.1. Plant materials

The materials included grass pea variety BioR-231 (diploid, 2n=2x=14), two types of induced auto tetraploid (2n=4x=28) lines designated as LsTI and LsTII (Lathyrus sativus tetraploid type I and II) and a triploid population (2n=3x=21) derived from diploid × tetraploid crosses [14, 15]. The two auto tetraploid lines differing in morphology, chromosomal configurations and segregation patterns have been maintained through self-pollination [15]. Fresh leaf samples from 10-d-old seedlings were used to analyze antioxidant defense activities.

2.2. Cu treatment

Dry and healthy seeds of three ploidy levels (last season harvest) were sterilized in 75% ethanol for 15 min, 2% sodium hypochlorite for 1 h, and thoroughly rinsed in sterile distilled water. Sterile seeds were scarified, soaked in distilled water for 12h, and germinated on moist filter papers in the dark at 25 °C. Five days after sowing seedlings from each ploidy group were transferred into 1L plastic pots containing Hoagland's nutrient solution with the addition of 50 mg L-1 CuCl2 for 9d under controlled conditions. All tests were replicated (5 plants/replication) thrice, using Cu-free nutrient solution as a control. All the solutions were refreshed at an interval of every three days with pH adjusted at 5.5±0.3.

2.3. Estimation of total and reduced ascorbate(AsA)

Total and reduced form of ascorbate were measured following the earlier methods [10].

2.4. Determination of antioxidant enzyme activities

Fresh leaf samples from primary branches were homogenized in an extraction medium containing 50 mM K-phosphate buffer pH 7.8, 0.1 mM EDTA, 2mM cysteine, 1% w/v PVP and 0.2% v/v Triton X-100. For the APX (EC 1.11.1.11) activity, 20 mM ascorbate was added to the extraction buffer. The extracts were filtered through two layers of cheesecloth, and the homogenate was centrifuged at 14000 g for 20 min, at 4°C. The supernatant fraction was filtered through a column containing 1 mL of Sephadex G-50 equilibrated with the same buffer used in homogenization. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm with extinction constant 2.8 mM-1 cm-1following the method of Nakano and Asada [16]. SOD (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium photochemical assay method as described by Beyer and Fridovich [17]. The reaction mixture (3ml) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 2µM riboflavin and 0.1 ml of enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction. DHAR (EC 1.8.5.1) were extracted with 50mM K- phosphate buffer (pH 7.8), 1% PVP-10, 0.2mM EDTA and 10 mM β-mercaptoethanol. DHAR activity was determined by ascorbate formation at 265 nm (ε = 14.1 mM-1 cm-1) for 3 min [16]. GR (EC 1.6.4.2) was extracted with the same medium as for DHAR but without β-mercaptoethanol and with 0.1% Triton X-100, and its activity was measured by monitoring glutathione-dependent oxidation of NADPH at 340 nm (ε = 6.22 mM-1 cm-1) for 3 min [18]. CAT (EC 1.11.1.6) was extracted in 50 mM K-phosphate buffer (pH 7.0) and 0.5% PVP-10, and its activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε = 39.4 M-1 cm-1) for 1 min [19]. Soluble protein content was determined according to Bradford [20] using BSA as a standard.

2.5. Membrane lipid peroxidation

Lipid peroxidation was determined by measuring the amount of malondealdehyde (MDA) following Talukdar [21]. Fresh leaf samples from different ploidy plants were cut into small pieces and homogenized in 5 mL of 10% trichloroacetic acid (TCA) solution with a mortar and pestle. Homogenates were centrifuged at 4,000 × g for 20 min. To each 2mL aliquot of the supernatant, 2 mL of 0.6% thiobarbituric acid (TBA) in (freshly prepared) 10% TCA solution was added. The mixtures were heated in boiled water for 15 min and then quickly cooled in an ice bath. After centrifugation at 4,000 × g for 10 min, the absorbance of the supernatant was recorded at 532 nm and 450 nm. Lipid peroxidation was expressed as the MDA content in nmol g-1 DW (dry weight).

2.6. Statistical analysis

All values are presented as means of at least three independent experiments with means ± standard error (SE). Multiple comparisons of means were performed using ANOVA, followed by Duncan's Multiple Range Test at $p < 0.05$.

[III] RESULTS

3.1. Levels of total and reduced ascorbate (AsA)

Usually, AsA content increased with ascending ploidy levels. Under Cu treatment, compared to respective controls, AsA redox declined sharply in Cu-treated diploids and triplo II, but varied marginally in triplo I and both tetraploids **[Table-1]**.

3.2. Membrane lipid peroxidation

Membrane lipid peroxidation, as measured by MDA content, was normal in diploid cells, but was increased 2.5-fold under Cu treatment. In leaves of both triploids, MDA content was lower than diploids under un-stressed condition. Upon exposure to Cu, there was no significant change of MDA level in triplo I, but MDA content increased sharply in triplo II. In tetraploids, MDA level was even lower than normal diploid level under Cu stress **[Table-1]**.

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Table: 1. Ploidy level variations in antioxidant defense response of *Lathyrus sativus* L. genotypes under Cu stress

Parameters	Diploids		Triplo I		Triplo II		LsT I		LsT II	
	0 mg/l (control)	40 mg/l	0 mg/l (control)	40 mg/l	0 mg/l (control)	40 mg/l	0 mg/l (control)	40 mg/l	0 mg/l (control)	40 mg/l
Reduced ascorbate (µmol g^{-1} dry weight)	2.78 ± 11e	0.76 ± 3.8f	4.03 ± 18c	3.95 ± 9.3c	3.33 ± 7.6d	1.12 ± 6.5 f	6.11 ± 19 a	5.95 ± 13 a	5.55 ± 21a	5.37 ± 26b
MDA (nmol MDA g^{-1} dry weight)	6.75 ± 0.78b	13.71 ± 1.4a	5.09 ± 0.67d	5.13 ± 1.2d	5.56 ± 0.79c	10.68 ± 2.1a	3.65 ± 0.56f	3.71 ± 0.77e	4.07 ± 1.4e	4.12 ± 1.3e
SOD [U mg^{-1} (protein)]	155.6 ± 4.5e	264.5 ± 6.0b	171.8 ± 3.8e	156.1 ± 2.5e	201.6 ± 4.1d	233.4 ± 4.5c	291.8 ± 7.3a	303.6 ± 8.1a	161.1 ± 4.8	311.8 ± 10.0 a
APX (nmol AsA oxidized min^{-1} mg^{-1} protein)	119.6 ± 3.9d	69.65 ± 4.8e	165.6 ± 4.1c	197.7 ± 5.3c	170.6 ± 3.8c	163.6 ± 5.5c	224.5 ± 10.7b	219.8 ± 9.0b	121.7 ± 5.1d	253.8 ± 6.0a
DHAR (nmol AsA formed min^{-1} mg^{-1} protein)	9.3 ± 1.2c	8.7 ± 0.89c	16.11 ± 0.87b	25.43 ± 5.4a	21.49 ± 4.7a	20.43 ± 4.0a	23.15 ± 3.8a	19.88 ± 2.5a	18.6 ± 3.3a	21.8 ± 4.0a
GR (nmol NADPH oxidized min^{-1} mg^{-1} protein)	22.67 ± 0.5c	10.34 ± 1.3d	45.31 ± 4.5b	52.34 ± 7.8a	57.67 ± 5.0a	60.63 ± 5.0a	67.73 ± 5.9a	63.69 ± 7.1a	45.28 ± 2.4b	51.61 ± 6.5a
CAT (nmol H_2O_2 degraded min^{-1} mg^{-1} protein)	41.34 ± 3.5c	23.17 ± 1.7d	43.88 ± 5.0c	57.56 ± 7.8b	55.53 ± 8.0b	60.83 ± 7.4b	29.76 ± 4.8d	32.15 ± 5.1d	39.87 ± 3.3c	77.83 ± 4.5a

The data are means ± SE of at least three independent experiments. Means followed by same letters are not significantly different by Duncan's Multiple Range Test at $P < 0.05$

3.3. Anti-oxidant enzyme activities

In the present study, activities of five prominent antioxidant enzymes namely SOD, APX, DHAR, GR and CAT varied in three different ploidy levels. In LsT I, both SOD and APX activities were 2-fold higher than those of diploids, whereas in LsT II, activities of SOD and APX were remained unchanged in relation to diploids **[Table-1]**. Activities of both DHAR and GR increased in both tetraploids over diploids; the increase was about 2.5-fold and 3.0-fold, respectively, in LsT I and about 2-fold for both enzymes in LsT II **[Table-1].** CAT activity was significantly ($P < 0.05$) lower in LsT I but was marginally changed in LsT II **[Table-1]**. The triploids derived from these two types of tetraploids also exhibited differences in antioxidant enzyme activities under un-stressed conditions. Triploids from LsT I (triplo I) showed that activities of all the five enzymes were intermediate between tetraploid and diploid parents **[Table-1]**. However, triploids obtained from LsT II (triplo II) exhibited rise of only DHAR and GR in comparison to triplo I **[Table-1]**. Significant alterations were recorded in their activities when 40 mg/l Cu-treatment was imposed. In diploid roots, SOD activity increased nearly 1.7-fold over control. APX, GR and CAT in diploids showed significantly downward trend under treatment, whereas changes in activities of DHAR between control and treated plants in diploid were statistically not significant. In triplo I, there was no significant change in SOD activity under treatment but APX, CAT, DHAR and GR registered a sharp increase over corresponding control values **[Table-1]**. By contrast, SOD level increased by 1.5-fold and no significant change in relation to control was observed for other enzyme activities in triplo II under Cu-treatment **[Table-1]**. Compared to control, no significant change was observed for the activities of five enzymes in LsT I but marked enhancement of SOD, APX and CAT activity was noticed in LsT II subjected to Cu treatment.

[IV] DISCUSSION

Response of grass pea genotypes to heavy metal induced stress was studied at diploid, triploid and tetraploid levels by applying high concentrations (40 mg/l) of $CuCl_2$. The effect of Cu was intimately associated with alterations in intrinsic biochemical mechanisms in leaves of grass pea plants at three ploidy levels. Cu is a redox-active material and thus, the main site of its attack in a plant cell is the cell membrane. Excessive concentrations of Cu are known to cause membrane lipid peroxidation through the generation of reactive oxygen species (ROS) [13, 21]. Accumulating evidences indicated that membrane lipid peroxidation could be used as a toxicity bioassay for plants, because it is very sensitive to Cu [21, 22]. In the present study, significant increase in MDA content in diploid leaf tissues of grass pea indicated Cu treatment induced oxidative damage in membrane through peroxidation of its lipid component. Significantly, MDA content was also very high in triplo II, but quite normal in triplo I and in both tetraploid types at 40 mg/l. The results strongly suggested onset of Cu-induced oxidative damage in diploids and triploids of LsTII with more severe effect on diploids and there was no symptom of oxidative stress in triploids from LsTI and both tetraploid types. The severity of membrane damage by lipid peroxidation was also reported in several edible grain legumes, including grass pea, lentil, fenugreek and common beans under cadmium and arsenic stresses [10-12, 23-27]. The situations as observed in the

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present material at three different ploidy levels can be better explained by considering AsA redox and responses of five prominent antioxidant enzymes to Cu-induced toxicity. Compared to control, SOD activity accelerated with high dose in diploid plants, indicating induction of its activity by Cu treatment and formation of excess superoxide radicals due to Cu treatment [27, 28]. SOD constitutes the first line of defense against free radicals, but its activity converts superoxide radicals into hydrogen peroxide, another ROS and signaling molecule within cell [8, 24, 25]. Efficient scavenging of H_2O_2 in the present diploid and triplo II leaves was badly impeded by below normal activity of APX and CAT, two major H_2O_2 -metabolizing enzymes in plants. Although mechanism and site of action of these two enzymes are quite different, complementation of these two enzymes during H_2O_2-scavenging was observed in grass pea mutants deficient in ascorbate and glutathione [10, 29]. In the present case, there was neither any scope of complementation nor the compensation of their actions in diplold and triplo II leaves as both of their activities declined sharply in response to Cu. Rising H_2O_2 has the capacity to inactivate these two enzymes [12, 26, 28, 30], might have resulted in oxidative damage of membrane lipids in Cu-treated diploid and triplo II plants. The low level of APX activity was also accompanied by significant decrease in GR. The reduction of GR level in both diploid and triplo II severely jeopardized the regeneration of reduced glutathione in the diploid plants, while ascorbate regeneration could not be enhanced due to failure of up-regulation of DHAR under Cu-induced stress. Declining levels of reduced ascorbate led to significant reduction of ascorbate pool in diploid and triplo II plants, which also have the potential to impede normal functioning of APX enzymes [10-12, 23-25, 30]. Overproduction of reactive oxygen species coupled with deficiency of antioxidant defense triggered high lipid peroxidation in diploid and triplo II leaves. By contrast, the normal level of SOD and increase in APX, CAT, DHAR and GR activities helped triplo I and both tetraploids in scavenging of reactive oxygen species including H_2O_2, by maintaining reducing environment of cell through the regeneration of reduced ascorbate and glutathione. Although glutathione content was not measured in the present material, high reduced ascorbate content led to significant rise in ascorbate redox state in triplo I and both tetraploid leaves. The results indicated differential responses of leaf antioxidant defense in different ploidy levels of grass pea. Even within the same ploidy level, reduced ascorbate content, enzyme activities and lipid peroxidation level differed significantly. It is also revealed that some of the enzymes were constitutively expressed at higher ploidy level, especially in tetraploids, but were induced in triplo I and LsT II. This suggested that the major portion of the antioxidative capacity of the tetraploids and triplo I was stimulated by Cu. All the five enzymes in tetraploid leaves scavenged ROS quite efficiently, resulting in normal level of MDA even at high Cu dose. This suggested normal functioning of ascorbate-glutathione cycle in harmony with other enzymatic machinery in detoxification of ROS in leaves of tetraploid grass pea plants. The better fitness of plants against abiotic stresses at higher ploidy level than diploids has also been reported in different crop plants [31, 32].

[IV] CONCLUTION

For the first time, antioxidant defense response in grass pea was studied at three ploidy levels under un-stressed control and Cu-treated conditions. The results revealed increase in antioxidant capacity in higher ploidy level than diploids and onset of Cu-induced oxidative stress in leaves of diploids. Significant differences regarding response of plants to Cu treatment at triploidy and tetraploidy suggested besides dosage increase, genomic stability may play vital role in tolerance of plants at higher ploidy levels. Both types of tetraploids were highly tolerant to Cu-induced oxidative damage, while among triploids, triplo I was comparatively higher tolerant than triplo II. It is also noteworthy that constitutive over-activity of GR and DHAR in tetraploids may be exploited to produce plant natural antioxidants in a commercial manner. Since polyploidization is generally accompanied by considerable morphological, cytological and/or physiological alterations and broader ecological amplitude, the knowledge gathered in the present investigation on better tolerance of triploid and tetraploid materials than diploids against Cu-induced stress may be exploited as a reference to unearth the mystery behind the adaptation of Lathyrus sativus and its related species in diverse ecological requirements for a long period of their domestication.

CONFLICT OF INTEREST

No conflict of interest in the form of either financial or commercial is involved in any way with the present study.

FINANCIAL DISCLOSURE

No financial sponsor in the form of person, institution or organization is involved in the present work.

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