



RESEARCH ARTICLE

FLOWER AND POD PRODUCTION, ABORTION, LEAF INJURY, YIELD AND SEED NEUROTOXIN LEVELS IN STABLE DWARF MUTANT LINES OF GRASS PEA (*Lathyrus sativus* L.) DIFFERING IN SALT STRESS RESPONSES

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ABSTRACT

Response of three stable dwarf mutant lines, namely *dwf1*, *dwf2* and *dwf3* to 170mM NaCl treatment was studied in comparison with untreated dwarf lines (positive control or PC) and treated mother varieties (used as negative control or NC) from 20d old seedling stage to maturity in grass pea (*Lathyrus sativus* L.). The three mutants differed significantly from both controls and also, from each other by onset of flowering, flower and pod production, their abortion, pollen fertility, different seed yield components, leaf injury level, plant dry weight and seed neurotoxin (β -ODAP) contents under salt stress. Among the five types of genotypes, *dwf1* and *dwf2* showed tolerance, while *dwf3* was considered as a salt-sensitive line. Among the five genotypes, NC plants were the least tolerance to NaCl treatment.

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INTRODUCTION

Soil salinity is one of the most severe abiotic stresses affecting production of the legumes worldwide (Bayuelo-Jiménez *et al.*, 2002; Wang *et al.*, 2003). This problem is more severe in arid and semi-arid regions, and legume plants already face or in the near future will face a notable impact of salt stress in these regions (Graham and Vance, 2003). Grass pea (*Lathyrus sativus* L.) is one of the oldest legume crops with cultivation period of more than 8000 years (Smartt, 1984). It can tolerate a wide range of biotic and abiotic stresses (Vaz Patto *et al.*, 2006), and is commonly

considered as moderate salt-tolerant (Campbell, 1997). Scanty information is available regarding the effect of salinity on grass pea with Mahdavi and Sanavy, (2007) tested four Iranian grass pea cultivars under salt stress at germination and seedling stage. No reports, however, are available regarding the effect of salinity on reproductive parameters, seed yield components and seed toxin levels. Low level of genetic variability in cultivated varieties and poor understanding of physiological parameters on its tolerance to environmental stresses are the two prime hindrances for fully exploitation of the remarkable potentiality of this crop through conventional breeding methods. Induced mutagenesis has been considered an effective tool to create additional genetic

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variability for different biotic and abiotic stress tolerance in legume crops (Vaz Patto *et al.*, 2006). During an ongoing research on development of different mutant stocks with desirable traits including abiotic stress tolerance, a number of valuable diploid mutant lines and different cytogenetic stocks have been isolated and characterized in grass pea in mutagen-treated population (Talukdar and Biswas, 2005, 2007; Talukdar, 2009b, 2010a). Among these mutants, three dwarf plant types, namely *dwf1*, *dwf2* and *dwf3* showing desirable variations in phenotypes, low seed toxin levels and differences in isozyme profiling have been characterized in detail, and genetic basis of their dwarfism has also been elucidated (Talukdar *et al.*, 2001; Talukdar, 2009a, 2010b). As salinity is an emerging problem in agriculture and biology, fitness of promising genotypes needs testing under salt stress before release. Like many other legumes, dwarf phenotype is a rare but agronomic desirable trait in grass pea, and the reproductive stage is most crucial as it ultimately determines grain yield. Moreover, preliminary studies pointed out significant variations of neurotoxin, β -N-Oxalyl-L α,β -diamino propionic acid (β -ODAP), content in response of water stress in seeds of different grass pea genotypes (Cocks *et al.*, 2000; Gengsheng *et al.*, 2001) Therefore, it is becoming increasingly important to test the growth performances, yield components and seed toxin levels in dwarf genotypes under salt-stress condition in grass pea. In the present investigation, performances under NaCl stress (watering with 170 mM NaCl-supplemented distilled water) of *dwf1*, *dwf2* and *dwf3* were studied at flowering to maturity stages. The main objectives of this study were to assess the variations in growth parameters, yield and seed toxin levels under high salt-stress regime at reproductive stages of growth in the three dwarf mutant lines of grass pea.

MATERIALS AND METHODS

Plant materials

Three dwarf mutant lines, namely *dwf1*, *dwf2* and *dwf3* were isolated in the genetic background of grass pea variety BioR-231 and Hooghly Local (HL), respectively. The *dwf1* was isolated in colchicine treated C_2 progeny of variety BioR-231,

while *dwf2* and *dwf3* were detected in 250 Gy and 300 Gy gamma ray induced M_2 progeny of variety BioR-231 and HL, respectively (Talukdar *et al.*, 2001; Talukdar, 2009a). The three mutant lines were true breeding and stable, and have been maintained for several generations by self-pollination.

Experiment set-up

Pot experiments were performed to test the salt stress responses of each of the three dwarf lines from seedling stage to maturity during winter season (November to February) of 2006-07, 2007-08 and 2008-09. The experiment was replicated thrice (30 plants mutant⁻¹replication⁻¹) in a completely randomized block design in each season. Salt treatment was commenced on 20 d old seedlings and continued up to maturity. Seeds were surface sterilized in 70% ethanol for 2 min, rinsed twice in de-ionized water and then placed on water-moistened filter papers in 9cm diameter Petridishes in an incubator at 25°C with 12h light following the guidelines of ISTA (2008) for germination during last week of October. Germinated seeds were immediately transferred to twelve inches earthen pots containing a mixture of fine soil, vermiculite and farm yard manure (1:1:1). The soil was classified as sandy loam with 6.9% clay, 20.8% silt and 72.3% sand with a neutral pH of 7.02.

Seedlings were thinned to two per pot after emergence and watered evenly for their uniform growth until 20d after first emergence. The pots were kept under control conditions with mean day/night temperature 27°C/20°C, 10h photoperiod and relative humidity of 77%±5%. Salt treatment was commenced on 20d old seedlings by watering the plants with 170 mM NaCl-supplemented distilled water thrice in a week. Untreated mutant plants given only distilled water were considered as positive control (PC), while the NaCl-treated mother varieties (BioR-231 and HL) were served as negative control (NC). Salt concentration was regularly checked by measuring electrical conductivity with a conductivity meter (Systronics M-308, Kolkata, India). Evapotranspirational losses were compensated daily with deionized water.

Morpho-physiological parameters

As mentioned earlier, the experiment was repeated in three consecutive seasons (cropping period November-February) with three replications season⁻¹ genotype⁻¹. However, no significant differences were found across seasons. Thus, mean value of three seasons is presented here. Each individual plant was measured for the following 17 parameters- days (after sowing-DAS) to flowering, days to 50% (DAS) flowering, number of flowers (primary and secondary) branch⁻¹, flower abortion (primary and secondary) branch⁻¹, pollen fertility (%), leaf injury level, days (after sowing) to maturity, number of pods plant⁻¹, pod abortion plant⁻¹, seed yield (g) plant⁻¹, 100 seed weight (g), seeds pod⁻¹, shoot and root dry weight (g) and seed neurotoxin level. The onset of flowering and podding date was recorded for each plant in both control and treatment.

New flowers were tagged, and flowering and subsequent podding date from primary and secondary branches were marked on the tags. Flower abortion was calculated from tags where no podding date was recorded. Pod abortion was calculated from tags where a podding date was recorded, but no pod was present or pods were present, but possessed small or no seed present at harvest (Leport *et al.*, 2006). To determine dry weights, after harvesting, plants were separated into roots and shoots. Roots were washed in tap water and rinsed in de-ionised water.

Plant materials were oven-dried at 65°C for 48h and weighed. After drying, tags and pods from primary and secondary branches were separated, and flower and pod abortion percentage were calculated. Mean values for number of flowers branch⁻¹, pods plant⁻¹, flower and pod abortion plant⁻¹ (%) and pollen fertility (%) was calculated at 15 d intervals from date of treatment imposition in both control and treated lines. Pollen fertility was determined by staining of freshly collected pollen grains with 1% acetocarmin solution following the procedure of Talukdar and Biswas (2007). Seed neurotoxin content was estimated at post-harvest stage using the colorimetric method of Rao (1978).

Determination of salt-induced injury level on leaf

Salt-induced leaf injury was rated on a scale from 0 to 4 based on injury gradation of Chen *et al.* (2007) with some modifications for *Lathyrus sativus* L.: 0: no salt injury (100% survival with no damage), 1: mild salt injury, indicated by small area (approximately 1/5) of leaflet apex and margin turning brownish yellow, 2: moderate salt injury, indicated by ½ of the leaflet turning whitish-yellow, 3: severe salt injury, when over 80% of total leaflet area turned whitish-yellow and very thin, and 4: extreme injury, when leaflets became crinkled, finally fell off and the plant ultimately died. Leaves from primary branches were considered for visual scoring of injury level.

Statistical analysis

The results are presented as mean ± standard errors. Statistical significance between mean values was estimated by simple 't-test' and ANOVA (STATISTICA 6.0, StatSoft, Inc,U.S.A.).

RESULTS

Phenology

A total of 20 plants (10 pots) mutant⁻¹ replication⁻¹ season⁻¹ was studied in three consecutive growing seasons to assess the salt-induced responses of the three dwarf mutants in grass pea. Barring days to end of flowering and seeds pod⁻¹, the three treated mutants (dwf1, dwf2 and dwf3) differed significantly from PC as well as NC and also from each other in their responses to salinity stress induced by 170 mM NaCl treatment on 20th day old seedlings up to maturity (Table 1). In comparison to PC plants, flowering commenced at 48 DAS in dwf1 and at 50 DAS in dwf2, a significant ($p < 0.05$) advance of 15 DAS and 13 DAS, respectively under salt treatment. Both the plant types reached 50% flowering stage within 10-14 days of onset of first flowering (60-62 DAS). Flowering period ended at 100 DAS in treated dwf1 and 118 DAS in dwf2 showing non-significant variations with PC. In salt-treated dwf3, flowering appeared at 40 DAS, and 50% flowering stage was achieved at 82

Table 1. Mean value (\pm SE) for 16 different traits in positive control (PC), three dwarf mutant lines and negative control (NC) of grass pea (*Lathyrus sativus* L.) at 170 mM NaCl treatment.

Traits ^a	PC ⁺	dwf1	dwf2	dwf3	NC ⁺	Calculated
						F-value
Days to first flower	63.0 \pm 0.44	48.0 \pm 0.32*	50.0 \pm 0.19*	40.0 \pm 0.21**	33.5 \pm 0.29***	*
Days to 50% flower	69.2 \pm 0.19	61.9 \pm 0.48	60.0 \pm 0.39	82.1 \pm 0.33***	44.5 \pm 0.53**	*
Days to end of flower	111.2 \pm 0.47	100.1 \pm 0.27	118 \pm 0.31	116 \pm 0.38	100 \pm 0.27	ns
Flowers branch ⁻¹ (pr)	6.6 \pm 0.10	10.7 \pm 0.17*	14.8 \pm 0.22*	3.1 \pm 0.07**	3.0 \pm 0.11**	*
Flowers branch ⁻¹ (sec)	6.3 \pm 0.15	9.8 \pm 0.36*	12.6 \pm 0.44**	2.1 \pm 0.09**	0.7 \pm 0.08***	*
Flower abortion (%)						
primary branch ⁻¹	5.8 \pm 0.03	7.3 \pm 0.14	7.0 \pm 0.16	83 \pm 0.21***	96 \pm 0.22***	*
secondary branch ⁻¹	9.0 \pm 0.07	10.0 \pm 0.08	11 \pm 0.13	95 \pm 0.10***	98 \pm 0.31***	*
Pollen fertility(%)	98.32 \pm 0.32	87.11 \pm 0.15	90.66 \pm 0.21	57.19 \pm 0.31**	41.78 \pm 0.89**	*
Pods plant ⁻¹	50.9 \pm 0.76	52.5 \pm 0.33	57.6 \pm 0.46	13.8 \pm 0.36***	9.6 \pm 0.29***	*
Pod abortion pl ⁻¹ (%)	0.02 \pm 0.03	0.06 \pm 0.09	0.01 \pm 0.06	10.0 \pm 0.16***	15 \pm 0.20***	*
Seeds pod ⁻¹	3.9 \pm 0.26	3.5 \pm 0.31	3.7 \pm 0.25	2.2 \pm 0.18*	1.3 \pm 0.20*	ns
Seed yield plant ⁻¹	11.6 \pm 0.63	8.96 \pm 0.47	10.3 \pm 0.36	1.2 \pm 0.15***	0.8 \pm 0.17***	*
100 seed weight	5.0 \pm 0.12	4.1 \pm 0.23	4.5 \pm 0.17	2.3 \pm 0.35**	1.9 \pm 0.44***	*
Shoot DW (g) plant ⁻¹	0.082 \pm 0.23	0.073 \pm 0.34	0.077 \pm 0.33	0.045 \pm 0.54**	0.030 \pm 0.32***	*
Root DW (g) plant ⁻¹	0.094 \pm 0.33	0.083 \pm 0.43	0.084 \pm 0.28	0.044 \pm 0.38***	0.028 \pm 0.21***	*
Seed neurotoxin (%)	0.17 \pm 0.43	0.13 \pm 0.29	0.11 \pm 0.18	0.45 \pm 0.49**	0.59 \pm 0.33***	*

*, ** and *** mean (n=20) value significant from PC (*t*-test) at $p < 0.05$, 0.01 and 0.001 levels, respectively; *genotypes differed significantly (ANOVA) at $p < 0.05$ levels; ns-not significant; PC⁺- untreated dwarf plants, NC⁺- NaCl treated mother varieties (BioR-231 and Hooghly Local), ^a Days calculated from sowing for onset of flower. 50% flower and end of flowering: pod number and abortion include both primary (pr) and secondary

DAS, whereas end of flowering was recorded at 116 DAS showing significant variations for the former two traits and non-significant for the later in comparison to PC plants. Flowering advanced in NC plants also, exhibiting highly significant ($p < 0.001$) variations from PC under NaCl-treatment (Table 1).

Flower and pod development, abortion and pollen fertility

The mean value for the duration of flowering (commencement-end) period was the highest in the salt-treated dwf3 plants (76d) in comparison to dwf1 (52d), dwf2 (68d), PC (48.2d) and NC plants (66.5d). Number of flowers borne on both primary and secondary branches in salt treated dwf1 and dwf2 plants surpassed PC significantly with usual number of pod set under salt stress (Fig.1-3, Table 1). However, as the treatment progressed, lesser number of flower and pod formations took place in salt treated dwf3 line (Fig. 1-3), and in comparison with PC plants, it registered 2.1-fold and 3.7-fold reduction during maturity, respectively. Reduction of flower was comparatively higher for secondary branches than those of primary branches (Table 1). The effect of salt treatment was more severe in NC

plants showing lowest mean values for flower and pod production (Fig. 1-3, Table 1).

The flower abortion percentage varied between 5.8% (PC) and 98.0% (NC) in five genotypes. It reached its highest level at 50% flowering stage on both primary and secondary branches in NC plants, and it was closely followed by dwf3 (83-95%). Abortion percentage, although not significant, peaked only at the end of the growing season in dwf1 and dwf2 lines (Fig. 4). As compared with primary branches, the effect was more pronounced on secondary branches (Table 1). As compared to other genotypes, pod abortion plant⁻¹ increased significantly ($p < 0.001$) in both dwf3 and NC plants as the treatment progressed (Fig. 5). Pollen fertility was as per PC level (98.32%) in dwf1 and dwf2, but it reduced 1.7-fold in dwf3 and 2.4-fold in NC plants (Table 1). Pollen fertility in dwf3 and NC plants decreased gradually with the progress of treatment from seedling stage to maturity (Fig.6).

Seed yield, seed weight and dry weight of plant organs

Mean values for seeds pod⁻¹, seed yield plant⁻¹, 100 seed weight and dry weight of shoot and root calculated from control and treated population at

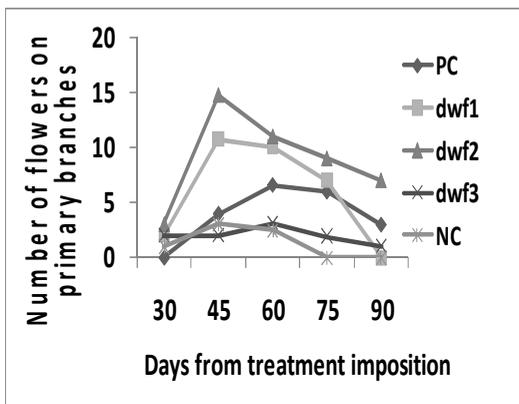


Fig. 1. Number of flowers primary branch

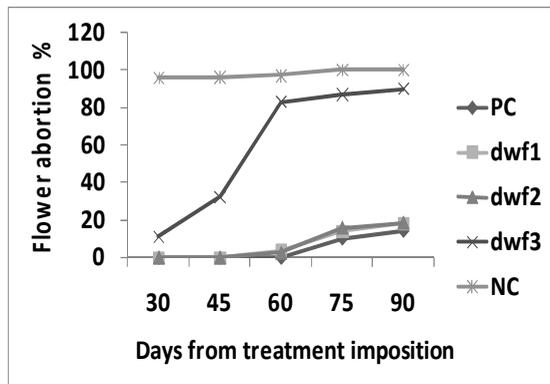


Fig. 4. Flower abortion (%)

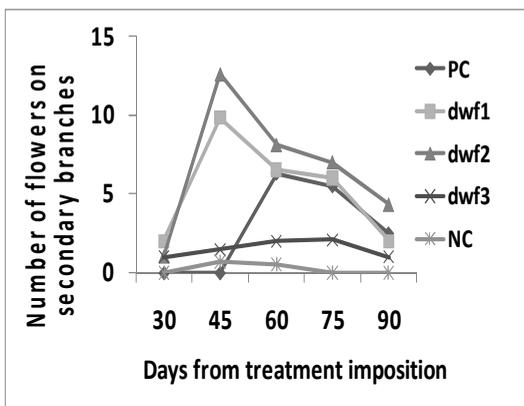


Fig. 2. Number of flowers secondary branch⁻¹

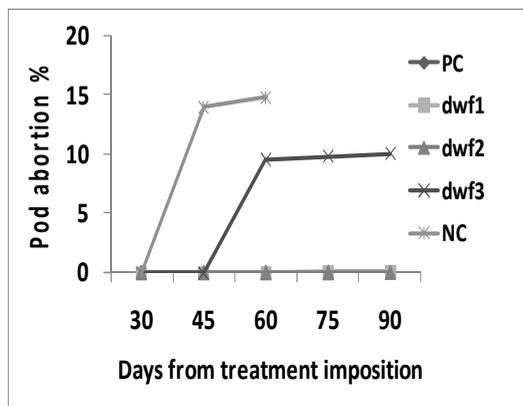


Fig. 5. Pod abortion

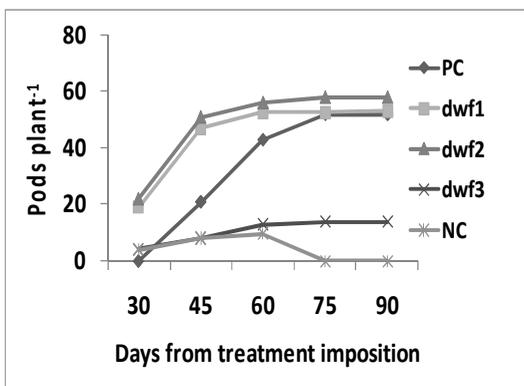


Fig. 3. Pods plant⁻¹

post-harvest stage decreased significantly in dwf3 and NC plants as compared with PC plants under salt treatment. The reduction was about 1.4-fold, 9.7-fold and 2.2-fold in dwf3, and 2.1-fold, 15-fold

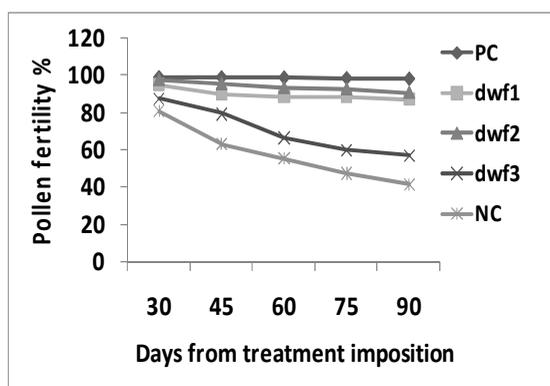


Fig. 6. pollen fertility

and 2.6-fold in NC plants for seeds pod⁻¹, seed yield plant⁻¹ and 100 seed weight, respectively (Table 1). In dwf3, shoot dry weight was reduced 1.8-fold, while dry weight of root decreased 2.1-

fold. In NC plants, reduction was much higher for shoot (2.7-fold) and root dry weight (3.4-fold). The change, however, was not significant in salt-treated dwf1 and dwf2 plants for the seed traits and dry weights (Table 1).

Salt-induced leaf injury

Salt-induced damage was marked by the contrasting modifications of green leaf portion to whitish-yellow colouration of about 50% of total leaflet area in dwf3 plants (injury level 2) and of about 80% of total leaflet area in NC plants (injury level 3). Destruction of normal colour was first noticed on the tip of the leaflet, which turned yellow and rapidly moved towards petiole. The injured portion became necrotic at later stages of growth. No trace of salt injury, however, was observed in leaves of dwf1 and dwf2 plants like non-treated PC plants (injury level 0).

Seed neurotoxin content under salt stress

Neurotoxin, β -ODAP, content was estimated from the seeds collected from tagged pods of treated and control plants in three consecutive seasons of experiment. Mean toxin level increased nearly 2.6-fold to 0.45% in dwf3 as compared with its PC (0.17%) under salt treatment. Toxin levels remained at control level in treated dwf1 and dwf2 types. In contrast, NC plants exhibited 3.5-fold increase in mean value of ODAP content (Table 1).

DISCUSSION

Grass pea (*Lathyrus sativus* L.), of the common legumes, is relatively salt tolerant, but no comprehensive strategy has been undertaken to improve the salt tolerance traits of this crop. Preliminary investigation revealed the potentiality of induced mutagenesis in developing genotypes with enhanced salt tolerance capacity through the generation of additional variability (D. Talukdar, unpublished observation). It was found that most of the grass pea genotypes could tolerate salinity level between 100-150 mM NaCl treatments. Therefore, a slightly higher concentration of 170 mM NaCl was selected in the present investigation to test the

responses of the three stable dwarf mutant lines of grass pea.

Flowering duration, flower and pod production, abortion and seed yield under salt stress

In the present investigation, performances of the three dwarf mutants, namely dwf1, dwf2 and dwf3 were studied from seedling stage to maturity mainly on the basis of reproductive growth potentials and seed characteristics in comparison to un-treated PC (mutants) and treated NC (mother varieties BioR-231 and HL) plants. All the five plant types varied significantly in their responses to high salt stress regimes. Salt treatment induced early flowering in three dwarf lines and NC plants in different magnitudes without any significant effect on end of the blooming period. This extended their flowering period considerably with the highest duration (76d) recorded in dwf3. However, salt treatment reduced flower number, pod production and increased their rate of abortion resulting in reduced seed yield in dwf3 line. This indicated that long flowering duration did not help dwf3 plants for achieving higher yield potential in stressed condition. It was important to note that flower and pod abortion was greater in late-produced flowers than in early-produced flowers. This was evidenced by the higher percentage of abortion in secondary branches than primary branches. Clearly, late onset of 50% flowering period led to higher abortion in dwf3 plants than dwf1 and dwf2, and as the treatment progressed it was becoming increasingly difficult for the treated dwf3 plants to maintain normal seed formation and grain filling. Further, seed size tended to decrease in late-set pods compared to early-set ones resulting in significant reduction of 100 seed weight in dwf3 plants. Reduced rate of pollen fertility and its gradual decrease with the progress of treatment also played a significant role in reduction of yield components in dwf3 plants. This performance was, however, better than NC plants which exhibited lowest mean values in different growth parameters with highest level of pollen sterility. Fewer numbers of viable pollen grains on receptive stigma, interference of sterile pollen grains with growth of pollen tube, shorter lifespan and reduced vigor of pollens may bring about disturbances in normal fertilization process leading

to pod abortion and concomitant decrease in grain yield in stress-sensitive genotypes (Akbar *et al.*, 1972; Porch and Jahn, 2001; Fang *et al.*, 2010).

In contrast, early flowering, quick onset of 50% flowering stage and high level of pollen viability throughout the treatment period helped both dwf1 and dwf2 plants to maintain normal flower and pod production by preventing significant loss due to flower and pod abortion at later stages of growth in stressed condition. The grain filling was also good as indicated by normal 100 seed weight value in these two lines under salt treatment. In different grass pea genotypes, seed yield was significantly affected by number of pods and/or number of seeds pod⁻¹, while pod number was positively correlated with number of flowers and their successful pollination (Talukdar, 2009c). Both flower and pod production and abortion were considered as the determining criteria for seed yield in chickpea under terminal drought (Leport *et al.*, 2006; Fang *et al.*, 2010). Apparently, these factors played significant roles in determining seed yield in the present dwarf lines, also. Reduction in flowers, pods and seeds pod⁻¹ under saline condition was also reported in different crops (Katerji *et al.*, 2001; Fang *et al.*, 2010).

Biomass allocation

Dry weight of plants was considered as one of the realistic criteria in determining salt responses in plants (Munns, 2005). Normal value for both shoot and root dry weight in dwf1 and dwf2 plants under salt treatment corroborates superior performance of these two dwarf lines. In contrast, lower mean values for dry weight and seed yield components indicated higher level of salt sensitivity in dwf3 line than PC as well as treated dwf1 and dwf2 lines. The detrimental effect of salt treatment was more severe on NC plants, which was suggested by the lowest means of dry weight. Interestingly, root development was more sensitive than shoot development in all the three salt-induced dwarf mutant lines, as indicated by lower mean value of root dry weight than shoot weight, and the result was in accordance with earlier reports in chickpea in salt-stressed condition (Ashraf and Waheed, 1993; Dua, 1997; Tejera *et al.*, 2006).

Salt-induced leaf injury

Leaf injury has been considered as one of the important salt response traits in plants (Munns, 2005; Chen *et al.*, 2007). On the basis of visual scoring of leaf injury on leaves borne on primary branches under salt stress, level 2 injury was manifested by dwf3 and level 3 by NC plants, indicating their susceptibility to salt treatment. Conspicuous absence of leaf injury in both dwf1 and dwf2 plants suggested greater level of tolerance of these two plant types to salt treatment. The cause of leaf injury was attributed to salt load exceeding the capability of leaf cells to compartmentalize salts in the vacuole. Salts would then build up rapidly either in the cytoplasm inhibiting enzyme activity or in the cell walls resulting in dehydration of the cell (Munns, 2005).

Salinity effect on seed neurotoxin levels

Several lines of evidences suggested accumulation of a neurotoxin, β -N-Oxalyl-L α,β -diamino propionic acid (β -ODAP) in response to abiotic stresses in seeds of grass pea (Cocks *et al.*, 2000; Gengsheng *et al.*, 2001). Lambein *et al.*, (1990) postulated that since ODAP is an amino acid it might have a role in the stress response that has made the grass pea such a useful species over the long period of its domestication. In the present study, significant increase in seed toxin content in dwf3 and NC plants and normal low level in dwf1 and dwf2 plants under saline condition suggested its over-accumulation as a symptom of stress in susceptible genotypes. This observation has immense significance in the sense that many improved grass pea varieties with low seed toxin content has been introduced in arid and semi-arid regions of different countries including India. As legume plants in these regions often face salinity and water stress at different levels, knowledge on sensitivity of seed toxin content in improved genotypes of grass pea to abiotic stresses is necessary for a comprehensive study. Hanbury *et al.* (1999) stated that genotype was the most important determinant of ODAP concentration and environment had less influence on seed ODAP concentrations. Under non-stressed environment, toxin content was generally higher (0.30%-0.39%) in dwf3 and mother varieties than dwf1 (0.14%)

and dwf2 (0.11%) in grass pea (Talukdar, 2009a). In the present study, over-accumulation of β -ODAP in seeds of dwf3 and NC plants and its non-significant change in dwf1 and dwf2 lines under saline condition indicated genotype-specific change of seed toxin content responding to NaCl treatment.

Salt stress response is a complex biological phenomenon. The present investigation revealed differential sensitivity among the three dwarf lines for important yield components and toxin content. The dwf1 and dwf2 line exhibited a fair level of tolerance, while dwf3 was highly sensitive to salt-induced changes. It is also worth mentioning that both the mother varieties, used as negative control, exhibited very high level of susceptibility to salt treatment with inferior performance of all the parameters even in comparison to dwf3 line. The information gathered in the present study can be used as a suitable reference to test the fitness of improved genotypes for broader introduction of grass pea in different geographical areas.

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