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ORIGINAL ARTICLE

Mitigating effects of salicylic acid against herbicidal stress

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Background, the context and purpose of the study: Pendimethalin [N-(1-ethyl propyl)-2, 6-dinitro-3, 4 xylidine] is one of the most commonly used herbicides. It induces harmful effect on non-target plants besides controlling the weed emergence. Salicylic acid (SA) plays an important role in abiotic stress tolerance. Present study was to assess the comparative efficacy of SA in combination with different concentrations of pendimethalin on black gram (*Vigna mungo*). The seeds of test plant were treated with field relevant concentrations (2, 5 and 10 ppm) of pendimethalin (P) and in combination with SA (0.5 mM) to observe effect of SA against herbicide toxicity. Experiment was performed in petri dish as well as in pot culture. The toxic effect of pendimethalin and SA on seed germination (SG), radicle length (RL) and mitotic index (MI) was evaluated in petri dish culture. Seedling height, pigments, protein, sugar contents and lipid peroxidation (LP) of 15 days old seedling were measured in pot culture. Total antioxidants (TA) were monitored as plant defence against oxidative stress.

Results, the main findings: Results showed that SG and seedling growth of *Vigna mungo* decreased under P_1, P_2 and P_3 treatments. RL and MI were also reduced significantly ($p<0.05$) in treatments with herbicide and reduction was more pronounced in P_3 treatment. A slight increase of SG and seedling growth was observed in P_2 treatment compared to P_1. Herbicide treatment remarkably declined pigment, protein and sugar contents of the seedlings when compared with control. TA and malondialdehyde (MDA) content increase significantly under pendimethalin treated seedlings. Combined treatment (P+SA) elevated growth of the seedlings. As a consequence of herbicidal stress, SA enhanced SG, RL, MI, pigment, protein and sugar content significantly. Under combined treatments, LP and TA were decreased when compared with pendimethalin treatment.

Conclusions, brief summary and potential implications: SA enhanced growth of *Vigna mungo* not only in combination with pendimethalin but also in treatment with SA alone as compared to control. Thus the results reveal, the role of SA in protection of *Vigna mungo* against herbicidal stress is apparent. The results are discussed in light of recent information.

Key words: Lipid peroxidation / Mitotic index / Pendimethalin / Total antioxidant / Vigna mungo

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Key words: Lipid peroxidation / Mitotic index / Pendimethalin / Total antioxidant / Vigna mungo

Abbreviations: P, Pendimethalin; SA, Salicylic acid; SG, Seed germination; RL, Radicle length; MI, Mitotic index; LP, Lipid peroxidation; MDA, Malondialdehyde; TA, Total antioxidant.

The widespread use of herbicides in modern agriculture resulted in environmental pollution, soil and water contamination. Environmental contaminations by these agents have impact on ecosystem. Their introduction into the food chain causes health problems to human and livestock. Pendimethalin, one of the most common pre-emergence herbicides and is used for control of

broadleaf weeds in crop fields (Sinha *et al*., 1996; Bhowmik and Ghosh, 2002). Pendimethalin belongs to di-nitroaniline group and has low water solubility, mobility and low volatile rate (Savage and Jordan, 1980; Schleicher *et al*., 1995). It may also cause severe damage to non-target plant species (Pahwa *et al*., 1988; Madhu *et al*., 1996). It is reported that pendimethalin causes reduction in root and shoot dry weight (Ashok *et al*., 1995) and inhibits germination and growth and imposes oxidative stress in crop plants (Smith, 2004). Superoxide radical produced due to oxidative stress causes damage to membrane by increasing lipid peroxidation (Smirnoff, 1993).

Salicylic acid (SA) one of the important plant signaling element plays major role in plants acclimation to stress (Durner *et al*., 1997). It plays an important role in abiotic stress tolerance. The considerable interests have been focused on SA due to its ability to induce a protective measure on plant under stress factors (Sakhabutinova *et al.,* 2003). SA is known to enhance antioxidative defense resulting into increased tolerance towards stress in plants. Exogenous SA application may be responsible for activation of defense genes (Tayeb *et al*., 2006).

The object of present study was to assess the mitigating properties of SA against herbicidal stress caused to the *Vigna mungo* seedlings by pendimethalin treatment. Seedling growth, pigment, protein, sugar and lipid peroxidation were examined. Total antioxidant was evaluated as plant defense against this stress.

MATERIALS AND METHODS

The certified seeds of *Vigna mungo* var. Shekhar were procured from seed agency at Allahabad. Healthy and uniform sized seeds were surface sterilized with 0.01% $HgCl_2$ solution and then rinsed five times with distilled water (DW).

Experiment 1

Seeds were treated with 2 (P_1), 5 (P_2) and 10ppm (P_3) concentrations of pendimethalin. Seeds were soaked in 0.5mM salicylic acid (SA) individually and also in combinations with pendimethalin. Seed germination, radicle length (RL) and cytological variations were recorded.

Cytological analysis

Root tips of seedlings were cut for cytological observations after 48 h of sowing and fixed in Carnoy's solution for 24 h and then transferred to 70% alcohol. The root tips were hydrolyzed in 1 N HCl for 20 min at room temperature and then stained with 2% acetocarmine solution for 1 h (Qian, 1998). Chromosome spreads were prepared by squash technique following Savaskan and Toker (1991). A total of 500 cells were scored from each preparation to study the mitotic index (MI).

Experiment 2

Seeds were sown in soil filled pots. Treatments as in experiment 1 were applied to the soil. Three replicates were used. Plants were watered as and when required. The seedlings were maintained in a growth chamber under controlled temperature (20±2°C), photoperiod of 16/8 hrs and photon flux density of 240 µ mol $m^{-2}s^{-1}$. Seedling height was recorded and first fully expanded leaves of 15 days old seedlings were taken for biochemical analysis.

Determination of leaf photosynthetic pigments and protein content

Chlorophylls and carotenoids were measured in fresh leaf samples. Leaf samples (10mg) were homogenized in 80% (v/v) acetone, filtered and then quantified spectrophotometrically according to Lichtenthaler (1987). Protein content was

determined following the method of Lowry *et al.* (1951) and amount of protein was calculated from standard curve obtained from bovine serum albumin.

Measurement of sugar content

Total soluble sugar was quantified according to Hedge and Hofreiter (1962). About 100 mg plant material was homogenized in 5 ml 95% ethanol. The homogenate was centrifuged at 4000*g* for 15 min. The supernatant (0.1ml) was mixed with 0.9ml DW and 4ml anthrone solution. The reaction mixture was boiled in water bath for 15 min. Absorbance was recorded at 620nm after cooling. Amount of sugar was calculated with reference to standard curve prepared from glucose.

Nitrate reductase

Nitrate reductase activity was assayed by the modified procedure of Jaworski (1971) based on incubation of fresh tissue (0.25 g) in 4.5 ml medium containing 100 mM phosphate buffer (pH 7.5), 3% KNO_3 and 5% propanol. Aliquot (0.4 ml) was treated with 0.3 ml 3% sulphanilamide in 3 N HCl and 0.3 ml 0.02% N-(1-napthyl)-ethylene diamine dihydrochloride (NEDD). The absorbance was measured at 540 nm. NR activity was calculated with a standard curve prepared using $NaNO_2$.

Lipid peroxidation

The lipid peroxidation in leaves was measured by determining the malondialdehyde content according to Heath and Packer (1968). The plant material (200 mg) was homogenized in 5 ml of 0.1% w/v trichloroacetic acid and centrifuged at 10,000*g* for 10 min. One ml of supernatant was mixed with 4 ml of 0.5% thiobarbituric acid (made in 20% trichloroacetic acid). The mixture was then heated at 95^0 C for 30 min and after cooling it was again centrifuged. The absorbance of supernatant was measured at 532 nm and corrected by subtracting the non-specific absorbance at 600nm. The MDA concentration was calculated using the extinction coefficient of 155 mM^{-1} and expressed as n mol g^{-1} FW.

Evaluation of total antioxidant capacity

The total antioxidant capacity of the plant extracts was evaluated by the method of Prieto *et al.* (1999). TA was quantified in a sample solution containing 0.1 ml of sample prepared by incubated 150 mg of plant material in 3 ml of ethanol, 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).The absorbance of the test sample was measured at 695 nm.

Statistical analysis

Statistical significance was assessed at the $p<0.05$ level using one way ANOVA and means were separated by Duncan's multiple range test ($p<0.05$) with the help of SPSS 10 software. Means and standard deviation were calculated from 3 replicates.

RESULTS

Seed germination (%), Mitotic index and Radicle length

The effect of pendimethalin and salicylic acid (SA) treatments on seed germination (SG), mitotic index (MI) and radicle length (RL) has been shown in Fig. 1. Herbicide significantly declined SG, MI and RL with maximum inhibition in P_3 treatment. SG and RL decreased in dose-dependent manner. SA promoted SG and RL to 4.01 and 96.73% over control. When SA applied with pendimethalin, SG and RL increased significantly as compared to pendimethalin treatment. MI was uniform under SA treatment but herbicide declines it in lowest and highest concentration with optimum at P_2.

Combined treatments have increased MI slightly when compared with pendimethalin treatments.

Pigment content

Herbicide decreased total chlorophyll. Amount of chlorophyll was maximum in SA treated seedlings. P_2 treatment was optimum for chlorophyll content. Combined effect increased total chlorophyll over pendimethalin treatment and the increase was not more than control or SA treatment. A different pattern was recorded in case of carotenoids. SA decreased (30.58%) carotenoids in comparison to control and was almost equal to P_1 and P_3 treatments. P_2 is optimum concentration and it increased carotenoids to the level of control. P+SA treatments enhanced the carotenoids content over SA, P_1 and P_3 treatments. (Table 1)

Protein, Sugar and Seedling height

Protein and sugar contents decreased successively with herbicidal stress. Maximum inhibition of 32.21 and 24.41% was observed under P_3 treatment in protein and sugar content, respectively. SA enhanced protein and sugar content over pendimethalin treated seedlings. Seedling height was not affected at SA and lowest concentration of pendimethalin. However, higher concentration of pendimethalin decreased seedling height with maximum inhibition of 17.54% was observed in P_3 treatment. A slight increase in seedling height of *Vigna* was recorded in combination of P+SA when compared with single treatments of pendimethalin. (Table 2)

Nitrate reductase, Lipid peroxidation and Total antioxidant

There was a significant ($p<0.05$) reduction in NR activity of the seedling in response to herbicide. The decrease was dose-dependent and maximum reduction of 81.89% observed in P_3 treatment. SA in single and in combined treatment promoted NR activity. LP and TA represent similar trends under respective treatments. MDA content declined slightly in SA treated seedling. However, pendimethalin progressively enhanced MDA content and maximum increase of 215.38% recorded in P_3 treatment. SA reduced LP when applied with pendimethalin and exhibited mitigating effect against herbicidal stress. TA was not influenced in SA treatment but increased under herbicide toxicity with maximum in P_3 treated seedlings. SA reduced TA when applied with higher concentration of pendimethalin. (Table 2)

Table 1 Effect of pendimethalin and salicylic acid on pigment contents in leaves of *Vigna mungo* L.

Treatments	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total Chlorophyll (mg/g FW)	Carotenoid (mg/g FW)
C	0.296±0.019ab	0.080±0.004ab	0.376±0.014ab	0.085±0.006a
SA	0.317±0.018a	0.097±0.024a	0.415±0.006a	0.059±0.005b
P_1	0.225±0.014cd	0.039±0.002de	0.257±0.016ef	0.062±0.005b
P_2	0.250±0.016bc	0.039±0.003cde	0.289±0.019de	0.089±0.005a
P_3	0.189±0.012d	0.029±0.002e	0.218±0.010f	0.061±0.002b
P_1+SA	0.251±0.018bc	0.071±0.002ab	0.322±0.0194cd	0.072±0.003ab
P_2+SA	0.292±0.006e	0.063±0.002bc	0.355±0.081bc	0.086±0.005a
P_3+SA	0.288±0.002ab	0.059±0.003bcd	0.348±0.005bc	0.084±0.005a

Mean±SE values followed by same letters are not significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3. C, control; SA, 0.5 mM; P_1, P_2, P_3 were 2, 5 and 10 ppm concentrations of pendimethalin respectively

Table 2 Effect of pendimethalin and salicylic acid on protein, sugar, nitrate reductase, lipid peroxidation and total antioxidant contents in leaves of *Vigna mungo* L.

Treatments	Protein (mg/g FW)	Sugar (mg/g FW)	Seedling height (cm)	Nitrate reductase (µmol NO2 g^{-1} FW h^{-1})	LP (n mol g^{-1}FW)	TA (Abs.)
C	104.60±0.30b	29.9±0.28ab	19.95±0.31a	14.25±0.60b	26.59±0.77f	0.43±0.11e
SA	107.15±0.20a	31.8±0.37a	20.25±0.14a	17.87±0.49a	22.75±0.1g	0.47±0.03e
P_1	93.23±1.36de	25.6±1.03d	18.35±0.66b	11.02±0.31d	57.06±0.68c	2.59±0.18bc
P_2	84.62±0.26e	24.3±0.51e	15.65±0.31cd	7.84±0.08f	75.31±1.16b	2.69±0.16b
P_3	70.90±0.98f	22.6±0.46f	14.65±0.20d	2.58±0.25g	83.86±0.27a	4.42±0.34a
P_1+SA	104.15±0.25c	27.4±0.46ab	19.25±0.14ab	12.09±0.17c	31.53±0.94e	2.45±0.49bc
P_2+SA	102.30±1.84cd	26.7±0.17b	17.00±0.86c	9.41±0.07e	33.03±2.40de	1.89±0.06cd
P_3+SA	97.25±0.89e	24.8±0.69c	16.45±0.02c	8.89±0.11e	35.97±1.01d	1.50±0.04d

Mean±SE values followed by same letters are not significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3. C, control; SA, 0.5 mM; P_1, P_2, P_3 were 2, 5 and 10 ppm concentrations of pendimethalin respectively

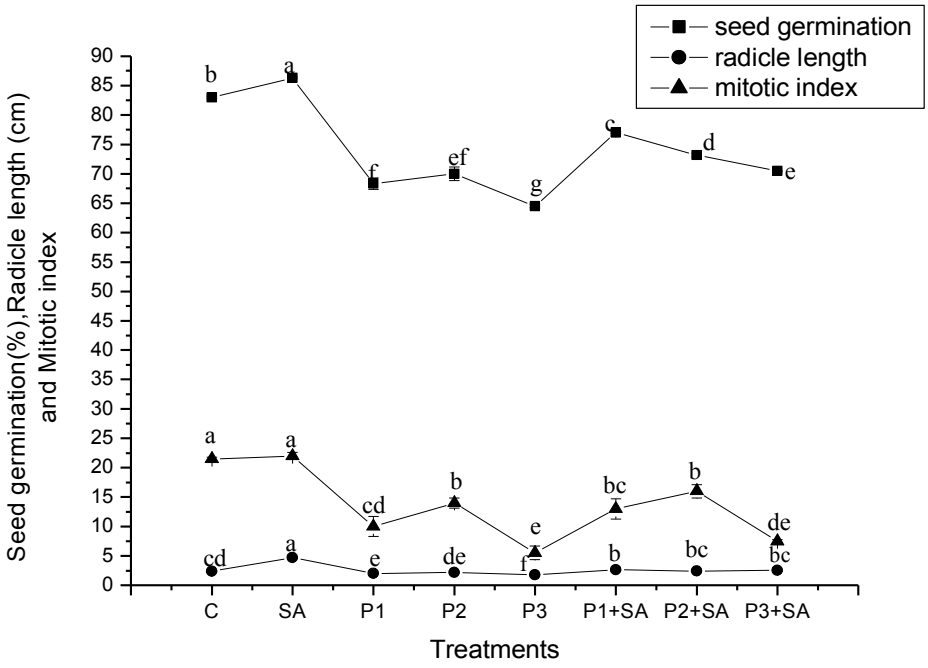

Figure 1: Effect of pendimethalin stress and salicylic acid (SA) on seed germination, radicle length and mitotic index of *Vigna mungo* L.

Mean±SE values followed by same letters are not significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3. C, control; SA, 0.5mM salicylic acid; P_1, P_2, P_3 were 2, 5 and 10 ppm concentrations of pendimethalin respectively.

DISCUSSION

Pendimethalin is a grass herbicide but effectively controls some annual broadleave plant. It also depresses non target plant species along with the weeds. Pendimethalin effect is usually noticed in form of inhibition of germination,

malformation (Lee *et al.*, 1998) and stunted of growth (Henderson and Webber, 1993). Our findings about germination, radicle length and seedling height clearly indicate that pendimethalin resulted in suppressed germination and growth of *Vigna mungo*. The reduction in SG, MI and RL was considerably higher in case of pendimethalin treatments which regulate the seedling growth. Chopra *et al*. (2009) reported that pendimethalin exert phytotoxic effects on crop by its downward movement to root zone in presence of sufficient moisture. SA induced SG and seedling growth of *Vigna mungo*. SA elevated RL by increasing cell division which is evident from MI. Chaoudhury and Panda (2004) reported that SA promoted RL in *Oryza sativa.* SA increased seedling height over control and in combined treatment as it mitigates the toxic effect of pendimethalin. Our results are in agreement with Coronado *et al*. (1998) who reported that sprayed aqueous solution of SA increased growth of shoot and roots in either greenhouse or field conditions.

The retarded germination, seedling growth and damaged roots due to herbicidal effect are the expression of altered metabolism associated with degradation of plant food reserve and absorption of minerals and water by roots. Protein and sugar contents decreased gradually with increased concentration of herbicide. It might be due to impaired photosynthetic machinery as result of herbicidal stress. Protein, sugar and pigment contents were enhanced in plants under SA treatment. Our results are in agreement with El-Tayeb *et al*. (2006) who reported promotary effect of SA on pigment, protein and sugar contents of sunflower.

The increased MDA content caused by pendimethalin is an indicator of lipid peroxidation and membrane damage. Malondialdehyde is decomposition product of polyunsaturated fatty acids (Lin and Kao, 2000). Positive and negative effects of synthetic herbicides on production of antioxidant compounds have been previously reported by several authors (Abu-Ismaileh *et al*., 1978; Rauchard *et al*., 1983). SA decreased LP and balanced total antioxidants to the level of control and thus promoted black gram growth. SA mitigated the toxic effect of pendimethalin during combinations by decreasing the MDA content and total antioxidant activity. Combination of SA+P led *Vigna* seedlings towards adaptation because TA decrease in those treatments, however promoted growth when compared with pendimethalin treatments. Plants develop several defense mechanisms to tolerate stress. Production of ROS scavengers i.e. antioxidant enzymes is an important tool to increased plant tolerance against oxidative stress (Sairam *et al*., 1998). Our results reveal improved performance of *Vigna mungo* in SA treatment when compared with pendimethalin and increasing adaptation when applied in combinations (P+SA).

CONCLUSIONS

SA enhanced growth of *Vigna mungo* not only in combination with pendimethalin but also in treatment with SA alone as compared to control. Thus role of SA in protection of *Vigna mungo* against herbicidal stress is apparent.

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