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ENHANCEMENT OF PEA PRODUCTION USING LEAF EXTRACT OF WITHANIA SOMNIFERA L. UNDER SUB-TROPICAL FIELD CONDITIONS OF KULLU, NORTHWESTERN HIMALAYA, INDIA

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□ *The present study investigates the promoting effects of leaf extract of* Withania somnifera *L plant on growth, biomass, and yield of* Pisum sativum *L plants. The growth and biomass of pea plants increased significantly over control due to leaf extract (LE-WS) treatment. Pea plants treated with LE-WS and ascorbic acid (L-AA) showed higher number as well as total dry weight of nodules over the control. The economic yield of pea plants due to LE-WS and L-AA treatments increased by 84 and 114%, respectively over control. The economic yield was significantly correlated with number of nodules ($R^2 = 0.75$, $P < 0.001$). The study suggests that LE-WS may be used to promote the growth and increase the production of pea plants in areas having multiple stresses. Detailed study on the use of extracts of medicinal plants as a tool to reduce the adverse effects of single stress is further required.*

Keywords: *Withania somnifera*, antioxidants, nodules, growth, biomass, economic yield

INTRODUCTION

Plants growing under natural conditions are inevitably exposed to various stresses. Oxidative stress generates free radicals (FR) or reactive oxygen species (ROS) under increasing stresses of drought, heat, chilling, UV-B radiations, nitrogen oxides (NO_x), sulfur dioxide (SO_2), ozone (O_3), heavy metals, pesticides, etc., leading to yield loss of economically important plants worldwide (Salin 1988; Agrawal et al., 2005; Tiwari et al., 2010; Sharma et al., 2010a). The free radicals can damage membrane lipids, proteins, pigments,

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and nucleic acids, lead to reduced growth and biomass productions and even death of some plants (Foyer et al., 1994; Sharma et al., 2010b). Earlier studies conducted in northwestern region of Indian Himalaya showed that the average concentrations of surface O_3, NO_X, and SO_2 ranged from 40.4–74.66, 1–7.6 and 7.3–21.4 μg m^{-3}, respectively during 1998–2002 and 2004 (Kuniyal et al., 2007a, 2007b). The atmospheric changes, i.e., O_3 transportations can produce transient O_3 peaks at concentration around 196.00 μg m^{-3} at sea level and more than 490.78 μg m^{-3} in the mountain regions (Davies and Schuepbach 1994). The increased in surface ozone at high altitudes can thus mainly due to the influence of photochemistry of locally produced air pollutants as well as their transportations from the nearby sources (Sunwoo et al., 1994). The vehicular emission and biomass burning for heating and cooking as well as forest fires are major contributors of the gases in Himalaya (Kuniyal, 2007b).

Increased ascorbic acid content in plants exposed to various stresses such as elevated carbon dioxide, ozone, heavy metals, etc., have been reported (Agrawal et al., 2005; Singh and Agrawal, 2007; Sharma et al., 2010a, 2010b) and can be considered as a multiple stress indicator. Ascorbic acid is itself an antioxidant and that accumulated in stressed plants. Therefore, in the present study, a synthetic antioxidant (ascorbic acid) is used as a positive control. The crude extracts of medicinal plants have been found to have strong free radical scavenging potential. Indian Himalayan Region is the rich source of medicinal plants and may have a strong antioxidant potential due to phenolic and flavonoid compounds (Raj and Shalini 1999; Tung et al., 2007). The secondary metabolites produced in plants have potential to reduce the adverse effects of these free radicals through scavenging them. Application of antioxidant rich extract of medicinal plants to a growth media of a plant may be used to reduce the effects of multiple stresses on the important agricultural crops.

A very few studies from India and abroad have been conducted on the use of the extracts of medicinal plants as a tool to neutralize adverse effects of multiple stresses at ambient levels on agricultural crops. In Himalaya, the elevated concentration of O_3 was reported, however their effects on crop yield is yet not studied. Earlier studies have shown that O_3 is one of the stress experienced by the plants over worldwide (Tiwari et al., 2005, 2010; Singh et al., 2010; Rai et al., 2007; Blum and Didyk 2006; Blum et al., 1997). Since antioxidants particularly natural antioxidant have been used to protect the crop yield loss due to elevated O_3 concentration (Blum and Didyk, 2007). Therefore, in the present study, an attempt was made to study the effect of leaf extracts of a medicinal plant (*Withania somnifera*) on growth, biomass and yield of pea (*Pisum sativum*), a sensitive leguminous crop to O_3 even at low concentration, exposed to ambient environmental conditions of mid Himalaya.

METHODS

Experimental Set Up

A pot experiment was carried out at G.B. Pant Institute of Himalayan Environment and Development, Himachal Unit, Mohal-Kullu (Lat. 31°58′00″ N, Long 77°06′04″ E and altitude 1,155 m) H. P., India during November 2009 to March 2010. The soil collected from the agriculture field was air dried, mixed uniformly with farm yard manure (soil and farm yard manure; 3:1). Two kg of mixed soil was filled into a pot of 20 cm height and 31 cm^2 area. A total of twelve pots were filled up and separated in three groups, i.e., four each. The first group was kept as control, whereas second and third groups were labeled as leaf extract and ascorbic acid, respectively. Each treatment had four replicates. The healthy seeds of the pea, procure from seed store, Bhunter, Kullu, Himachal Pradesh were manually identified and kept in water for three hours and then washed with double distilled water and air dried for two hours before sowing. Five seeds were hand sown at 1cm depth in each pot at the equal distance and 10 days after germination (DAG), plants was thinned to three plants per pot. The manual weeding was performed as and when required. The uniform moisture was maintained through supplying a constant volume (1 L pot^{-1}) of irrigation water.

Collection of Plants, Extract Preparation and Supplementation

Awagandha *(W. somnifera)* plants growing in the campus of G.B. Pant Institute of Himalayan Environment and Development, Himachal Unit, Mohal-Kullu, Himachal Pradesh, India. were selected for the present study and were collected. The whole plants were washed with running tap water to remove the adhered soil and dust particles. Roots, stems and leaves were separated manually. The plant parts were air dried and converted into powder using a grinder and the powder was passed through a 2-mm pore size sieve. 1g of root/stem/leaf powder was taken in a 100 mL conical flask and 25 mL of 80% methanol was added, shaken vigorously and kept at 4°C for 24 hours. The supernatant was separated and the total volume was maintained to 25 ml using 80% methanol. The total phenolic and flavonoid contents were measured in the extracts of roots, stems and leaves of *W. somnifera* plants using the modified methods of Wolfe et al. (2003) and Ordon-Ez et al. (2006), respectively. The phenolic concentration was calibrated in relation to tannic acid and flavonoid concentration was correlated in relation to quercetin. Since the antioxidant content was found markedly higher in the leaf extracts of *W. somnifera* plant. Therefore, leaf extract was used for the further study.

Each pea plant of respective treatments was supplemented with 10 mL of the leaf extracts (LE-WS, 0.04%) and L-ascorbic acid (L-AA, 0.01%) after 15 DAG of age. The plants under control i.e., exposed to multiple stresses were supplemented with same volume of double distilled water. The treatments were repeated at the intervals of 10 days up to 80 DAG of the plants age.

Sampling and Analyses

The three and six plants from each treatment were sampled at 45 and 90 DAG of the plant's age, respectively. The plants were washed with running tap water and air dried for two hours. The number of leaves, nodules, nodes, and root and shoot lengths at both 45 and 90 DAG, number of pods, length of pods and number of seeds per plant at 90 DAG of the plant's age were measured manually. The whole plants were separated in nodules, roots, stems, green and yellow leaves and were weighed to obtain the fresh weight. Plant's parts were oven dried separately at 80°C till a constant was achieved.

The plant parts weighed separately and added for total plant dry weight. The fresh weight of seeds of the pea plants per pot was considered as economic yield. The economic yield was calculated and expressed in term of kg ha^{-1}. Root shoot ratio (RSR) was calculated by dividing the root dry weight with that of shoots of pea plants. The harvest index (HI) was also calculated by dividing the dry weight of edible portion with that of aerial portion of the pea plants.

The significant differences between the treatment means were tested using Duncan's multiple range test at significant levels $P < 0.05$. Two-way ANOVA test was also performed to test the effects of different variables such as treatments, age and age × treatment on the tested parameters of the pea plants. All the statistical analyses were performed by using SPSS software version 12 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Total Phenolic and Flavonoid Contents in the Extracts

The total flavonoid contents in the methanol extracts of leaves of *W. somnifera* plant was significantly higher as compared to their root and stem extracts (Table 1). However, total phenolics in methanol extracts of roots

TABLE 1 Amounts of total phenolic and flavonoid contents in methanol extracts of the dry roots, stems and leaves of *W. somnifera* plants

Extract	Total phenolics (mg tannic acid/g)	Total flavonoids (mg quercetin /g)
Roots	$9.26^{a} \pm 0.20$	$0.25^{b} \pm 0.01$
Stems	$6.09^{b} \pm 0.14$	$0.46^{b} \pm 0.02$
Leaves	$9.64^{a} \pm 0.04$	$3.92^{a} \pm 0.12$

Values (mean of three replicates ± 1S.E.) in each row of the respective parameter, followed by same letters are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test).

and leaves were significantly higher as compared to that of stem. These results clearly showed that the leaf extract of *W. somnifera* plants have more antioxidant potential over the stems and roots.

Growth Performance

The physical appearance of 45 DAG old pea plants is shown in Figure 1. Overall, no visible symptoms or injuries were observed in any of the treated pea plants during its growth and development. However, insect feedings and leaf senescence were observed more in control as compared to L-AA and LE-WS treated pea plants. A better growth of pea plants over control was observed in plants supplemented with either LE-WS or L-AA. The growth performance of pea plants treated with LE-WS and L-AA were further evaluated in term of number of leaves, nodes and nodules, and roots, shoots and total plant length (Table 2). The above parameters increased significantly with the age of plant. The number of leaves, shoots and total plant length in the pea plants was found maximum and significant due the treatment of LE-WS and L-AA at 45 and 90 DAG, respectively. The root length was found to be decreased due to LE-WS and L-AA treatments significantly as compared to control at 45 DAG, whereas maximum root length was recorded in LE-WS treated plants at 90 DAG, followed by L-AA treated and control plants (Table 2). The number of nodes did not show any significant change due to the treatments (Table 2). The number of nodules per plant at both harvests was increased by the LE-WS and even more by L-AA (Table 2). The results of two-way ANOVA test further showed that the variables such as age, treatments and their interaction have significant effects on the tested parameters, whereas treatment and interaction of treatment and age did not have significant effects on number of nodes (Table 2). From this result, it is clear that LE-WS can significantly improve the growth characteristics of the pea

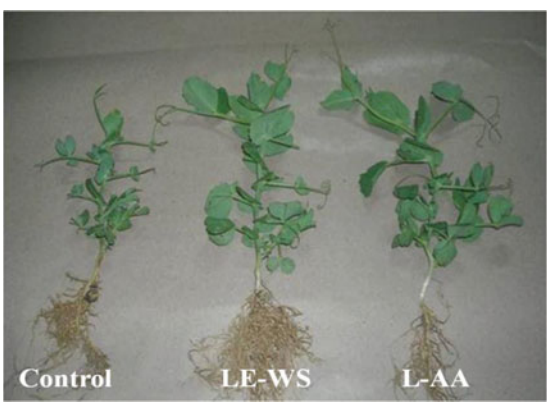

FIGURE 1 *P. sativum* plants exposed to multiple stresses (control), methanol extracts of leaf of *W. somnifera* and L-ascorbic acid (positive control) (Color figure available online).

TABLE 2 Effect of methanol extracts of the leaf of *W. somnifera* plants and L-ascorbic acid on growth characteristics of *P. sativum* plants

		Treatment		
Plant age (DAG)	Growth parameter	Control	LE-WS	L-AA
45	No. of leaves (Plant^{-1})	$30.67^{c} \pm 1.45$	$41.67^{a} \pm 0.88$	$36.00^{b} \pm 1.53$
	No. of nodules (Plant^{-1})	$39.33^{c} \pm 1.76$	$54.33^{b} \pm 1.86$	$85.00^{a} \pm 5.00$
	No. of nodes (Plant^{-1})	$8.00^{b} \pm 0.00$	$8.33^{b} \pm 033$	$9.33^{a} \pm 0.33$
	Root length (cm plant^{-1})	$19.57^{a} \pm 0.23$	$12.77^{b} \pm 0.22$	$12.67^{b} \pm 1.33$
	Shoot length (cm plant^{-1})	$14.33^{c} \pm 0.88$	$27.30^{a} \pm 1.53$	$22.73^{b} \pm 1.53$
	Total plant length (cm plant^{-1})	$33.90^{b} \pm 0.78$	$40.07^{a} \pm 0.55$	$35.40^{b} \pm 0.67$
90	No. of leaves (Plant^{-1})	$94.17^{c} \pm 2.66$	$111.00^{b} \pm 3.86$	$121.33^{a} \pm 2.97$
	No. of nodules (Plant^{-1})	$59.50^{b} \pm 1.78$	$100.83^{a} \pm 2.50$	$124.83^{a} \pm 16.82$
	No. of nodes (Plant^{-1})	$13.67^{ab} \pm 0.95$	$14.17^{a} \pm 0.75$	$11.17^{b} \pm 0.83$
	Root length (cm plant^{-1})	$20.50^{b} \pm 0.56$	$24.67^{a} \pm 1.02$	$21.00^{b} \pm 0.77$
	Shoot length (cm plant^{-1})	$29.17^{b} \pm 1.14$	$28.00^{b} \pm 0.93$	$36.83^{a} \pm 1.35$
	Total plant length (cm plant^{-1})	$49.67^{b} \pm 1.31$	$52.67^{b} \pm 1.50$	$57.83^{a} \pm 1.76$
Results of ANOVA test (F-value)				
		Age (A)	Treatement (T)	A × T
	No. of leaves (Plant^{-1})	1843.83***	32.61***	8.66**
	No. of nodules (Plant^{-1})	76.89***	60.15***	4.77*
	No. of nodes (Plant^{-1})	23.67***	0.35^{NS}	1.83^{NS}
	Root length (cm plant^{-1})	71.71**	5.12*	15.27**
	Shoot length (cm plant^{-1})	105.07***	24.71***	22.79**
	Total plant length (cm plant^{-1})	243.35***	8.32**	6.59*

Values (mean of three replicates ± 1S.E.) in each row of the respective parameter, followed by same letters are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Variable F-values are followed by the levels of significance: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, NS = Not significant.

plants exposed to ambient climatic conditions in Himalaya. Earlier studies have also shown that the extracts of medicinal plants have improved vegetative characteristics of potato plants (Abd-El-Kareem et al., 2001; Abd-El-Khair and Haggag, 2007). The increased growth of pea plants in the present study resulting from the treatments of LE-WS and L-AA may be ascribed to increase nitrogen fixation resulting from increased nodule formation during the growth and development of pea plants.

Fresh and Dry Matter Productions

The fresh and dry matter productions due to treatments of both LE-WS and L-AA, and the results of two-way ANOVA tests are given in Table 3. The results showed that the fresh and dry matter production in all the treatments increased with the age of plant. Fresh and dry weights of total plant were significantly higher in treated plants as compared to the control at both harvests (Table 3). The dry weight of total plants of pea increased by 73 and 83% due to LE-WS and 90 and 39% at 45 and 90 DAG, respectively over control plants (Table 3). No significant difference between the mean values

TABLE 3 Effect of methanol extract of the leaf of *W. somnifera* L. plants and L-ascorbic acid on biomass accumulation in *P. sativum* L. plants

		Fresh wt. (g plant^{-1})			Dry wt. (g plant^{-1})		
Plant age (DAG)	Parameter	Control	LE-WS	L-AA	Control	LE-WS	L-AA
45	Root	$0.82^b \pm 0.04$	$0.63^c \pm 0.02$	$1.42^a \pm 0.07$	$0.20^b \pm 0.01$	$0.17^b \pm 0.01$	$0.24^a \pm 0.02$
	Nodule	$0.06^b \pm 0.00$	$0.13^b \pm 0.01$	$2.31^a \pm 0.10$	$0.03^b \pm 0.00$	$0.05^b \pm 0.00$	$0.30^a \pm 0.04$
	Stem	$0.86^b \pm 0.03$	$1.30^a \pm 0.09$	$1.15^a \pm 0.06$	$0.12^b \pm 0.01$	$0.26^a \pm 0.03$	$0.17^a \pm 0.01$
	Leaf	$1.06^b \pm 0.08$	$2.80^a \pm 0.28$	$2.31^a \pm 0.10$	$0.18^c \pm 0.01$	$0.44^a \pm 0.04$	$0.30^a \pm 0.04$
	Total plant	$2.80^b \pm 0.15$	$4.86^b \pm 0.40$	$7.19^a \pm 0.33$	$0.53^b \pm 0.03$	$0.92^a \pm 0.08$	$1.01^a \pm 0.11$
90	Root	$0.29^b \pm 0.02$	$0.25^b \pm 0.02$	$0.48^a \pm 0.07$	$0.27^b \pm 0.02$	$0.31^{ab} \pm 0.03$	$0.37^a \pm 0.03$
	Nodules	$0.15^b \pm 0.02$	$0.27^a \pm 0.01$	$0.26^a \pm 0.01$	$0.09^b \pm 0.00$	$0.15^a \pm 0.01$	$0.15^a \pm 0.01$
	Stem	$3.27^b \pm 0.25$	$5.85^a \pm 0.69$	$5.33^a \pm 0.08$	$1.44^a \pm 0.17$	$1.39^a \pm 0.14$	$1.30^a \pm 0.08$
	Leaf	$5.62^a \pm 0.51$	$6.87^a \pm 0.90$	$5.18^a \pm 0.66$	$1.61^b \pm 0.17$	$2.79^a \pm 0.17$	$1.61^b \pm 0.12$
	Total plant	$24.25^b \pm 0.8$	$40.96^a \pm 1.62$	$44.52^a \pm 0.82$	$6.79^c \pm 0.36$	$12.64^a \pm 0.35$	$9.43^c \pm 0.24$
Results of ANOVA test							
		Age (A)	Treatments (T)	A × T	Age (A)	Treatments (T)	A × T
	Root	189.23***	38.98***	18.19***	19.31**	5.76*	0.71^{NS}
	Nodules	294.55***	476.14***	408.21***	0.08^{NS}	43.51***	21.29***
	Stem	53.55***	4.91*	3.02^{NS}	96.99***	0.07^{NS}	0.69^{NS}
	Leaf	47.23***	5.30*	1.95^{NS}	153.05***	13.53**	7.15**
	Total plant	414.66***	25.88***	16.27***	365.67***	15.18***	11.28**

Values (mean of three replicates ± 1S.E.) in each row of the respective parameter, followed by same letters are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Variable F-values are followed by the levels of significance: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, NS = Not significant.

of above tested parameters due to LE-WS and L-AA was recorded. However, a significant difference due to the treatments of both LE-WS and L-AA over control was recorded (Table 3). Leaf fresh weight varied significantly due to the treatments at 45 DAG, whereas did not change significantly at 90 DAG of the plants. The results of two-way ANOVA test showed that age, treatment and their interaction have significant influence on fresh and dry matter productions of tested parameter of the pea plants (Table 3). The interactive effects of age and treatments on fresh weight of leaf and stem and dry weights of roots and stems were found insignificant. The results further showed that L-AA increased fresh and dry weights of nodules per plants at 45 DAG, whereas LE-WS did not and both LE-WS and L-AA increased fresh and dry weights of nodules per plant at 90 DAG.

Yield Attributes

The effects of one-way ANOVA test and different treatments of the LE-WS and L-AA on the yield attributes of the pea plants are presented in Table 4. The number of pods did not vary due to the treatments, whereas number of seeds were not significantly higher in L-AA than in the LE-WS treatment. Pod dry weight, seed fresh and dry weights varied significantly in all the treatments as compared to the control plants (Table 4). The percent increments of the dry weights of pods and seeds over control were found as 140 and 130 by LE-WS and 88 and 70 by the L-AA, respectively (Table 4). The increase in dry weights of pods and seeds may also be ascribed to the antioxidant activities of leaf extracts as well as ascorbic acid.

TABLE 4 Effect of methanol extracts of the leaf of *W. somnifera* plant and L-ascorbic acid on pod and seed characteristics, and economic yield of *P. sativum* plants

		Treatment		
Yield attributes	Control	LE-WS	L-AA	Significance level
		Pod characteristics		
Number (plant^{-1})	$5.17^{a} \pm 0.48$	$5.33^{a} \pm 080$	$6.33^{a} \pm 0.61$	0.96^{NS}
Length (plant^{-1})	$8.63^{ab} \pm 0.29$	$8.17^{b} \pm 0.48$	$9.23^{a} \pm 0.08$	2.71^{NS}
Fresh wt. (plant^{-1})	$8.25^{c} \pm 1.04$	$19.00^{a} \pm 1.15$	$15.22^{b} \pm 0.99$	26.35^{***}
Dry wt. (g plant^{-1})	$2.02^{c} \pm 0.35$	$4.84^{a} \pm 0.59$	$3.44^{b} \pm 0.32$	10.29^{**}
		Seed characteristic		
Number (plant^{-1})	$27.67^{b} \pm 1.12$	$35.00^{ab} \pm 4.85$	$39.17^{a} \pm 2.52$	3.27^{NS}
Fresh wt. (plant^{-1})	$6.67^{b} \pm 0.41$	$12.29^{a} \pm 0.48$	$14.28^{a} \pm 1.21$	25.12^{***}
Dry wt. (g plant^{-1})	$1.37^{c} \pm 0.14$	$3.16^{a} \pm 0.24$	$2.57^{b} \pm 0.17$	23.34^{***}
Economic yield (kg ha^{-1})	$694^{b} \pm 42$	$1279^{a} \pm 50$	$1486^{a} \pm 125$	26.35^{***}

Values (mean of three replicates ± 1S.E.) in each row of the respective parameter, followed by same letters are not significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test). Variable F-values are followed by the levels of significance: $*** = P \leq 0.001$, $** = P \leq 0.01$, NS = Not significant.

Root Shoot Ratio and Harvest Index

The RSR was found significantly minimum in LE-WS treated pea plants, whereas markedly higher harvest index was recorded (Figure 2). The harvest index was only higher than the control in the L-AA and not in the LE-WS treatment. These results clearly showed that LE-WS treatment allocated more biomass accumulation to the above ground part and further into the edible portion, i.e., seeds of the pea plant as compared to the control and L-AA treated plants.

Economic Yields

The economic yield, i.e., fresh weight of seeds of pea plants treated with LE-WS was significantly higher (84%), followed by L-AA (114%) over control. Thus the results clearly showed that free radicals produced under the ambient conditions of Kullu were more neutralized by the L-AA as compared to the methanol extracts of leaves of *W. somnifera* plant during growth, development and seed formation of the pea plants (Table 4). The presence of various active compounds in the leaf extracts of *W. somnifera* plants which may lead to scavenging of different types of reactive oxygen species or free radicals produced in pea plants during growth and development. The increased economic yields of pea plants with application of leaf extract and ascorbic acid are likely due to their antioxidant activities. A positive and significant relationship between the number of nodules and HI with the economic yield at 90 DAG observed was also further observed, ($R^2 = 0.75$, $P < 0.001$; $R^2 = 0.65$, $P < 0.001$, respectively) indicating that more nitrogen uptake by pea plants, biomass allocation to seeds increased with increasing concentration of antioxidant potential of the pea plants. The economic yield

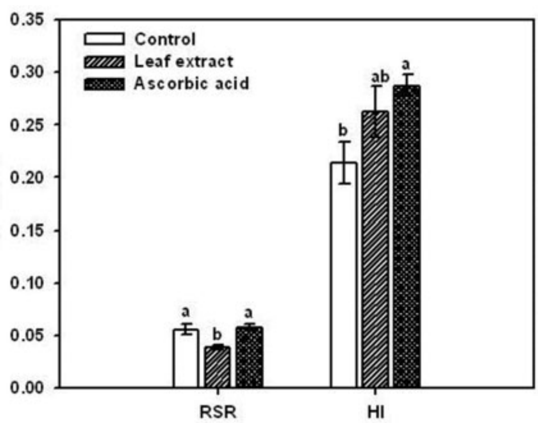

FIGURE 2 Effect of methanol extracts of the leaves of *W. somnifera* plants on root shoot ratio (RSR) and harvest index (HI) of *P. sativum* plants.

of potato and pigeon pea were increased by the applications of the extracts of medicinal plants (Abd-El-Kareem et al., 2001; Abd-El-Khair and Haggag 2007) and of *Tephrosia* (Minja et al., 2002). The increased economic yield of pea plants may also be ascribed to reduced effects of insect feedings and other fungal diseases in the presence of leaf extracts and ascorbic acid.

CONCLUSIONS

The study concludes that the methanol extracts of *W. somnifera* plants have significant potential to neutralize the negative and adverse effects of the multiple stresses, may be O_3 on pea plants during its growth and development and consequently increased the economic yield, under the field conditions of Himalaya. Treatments of pea plants with leaf extracts and ascorbic acid have further increased the number of nodules and its dry weights, which consequently increased the economic yield of the pea plants through modifying the nitrogen uptake due to their activities. Thus the study suggests that the methanol extracts of *W. somnifera* plants could be used as a management tool to reduce the adverse effects of ambient stresses on pea crops in Kullu Himalayan Region and its production could be improved. An in-depth study on the use of medicinal plant extracts as a tool against reducing the adverse effects of single or a specific stress is further required.

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