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Allelopathic activity of *Nicotiana plumbaginifolia* at various phenological stages on sunflower

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ABSTRACT

The leachates from different phenological stages (vegetative, flowering and fruiting) of *Nicotiana plumbaginifolia* decreased the growth and biochemical parameters of sunflower (*Helianthus annuus* cv. PAC-36) seedlings. In Petri plate assays, the leachates inhibited the germination and seedling growth of sunflower. In soil culture, the leachates decreased the pigments contents, proteins and the nitrate reductase activity. The decrease was maximum in seedlings treated with leachate from the flowering stage and was concentration dependent. Leachates also caused the oxidative stress and stimulated the activities of superoxide dismutase (SOD) and catalase (CAT). However, SOD and CAT activities inhibited the seedlings growth, when treated with the highest concentration of leachate from the flowering stage. Impaired metabolic activity due to leachates decreased the root and shoot lengths of sunflower. *N. plumbaginifolia* at the flowering stage was most phytotoxic to sunflower.

Key words: Allelochemicals, bioassay, catalase, chlorophyll, *Nicotiana plumbaginifolia*, phenological stages, superoxide dismutase.

INTRODUCTION

In nature, chemical warfare exists among plants. The chemicals involved in the process are known as allelochemicals. Allelochemicals are secondary metabolites produced by plants and are byproducts of primary metabolic processes. *Nicotiana plumbaginifolia* Viv. is a common solanaceous weed which grows from July to March in Uttar Pradesh and other parts of North India. It is rich in allelochemicals and contains several phenolic, polyphenolic and tannin compounds, in form of glycosides. The principal polyphenols are: rutin (quercitin-3-rhamnosidoglucoside), chlorogenic acid (3-caffeoylquinic acid) and their isomers. Other polyphenols are: quinic acid, shikimic acid, quercitin, isoquerciterin, scopoletin (7-hydroxy-6-methoxy coumarin) and kaempferol glycosides. Besides, it also contains several pyridine alkaloids of which nicotine (β-pyridyl-α-N-methyl pyrrolidone) and nor-nicotine are most important (3). Production of allelochemicals in plants is influenced by various abiotic and biotic factors [light (44), nutrients (29), water stress (17), extreme temperatures (44), herbicide and pesticide treatments (42), genotype (27) etc]. Besides, the phenological stage also influences the

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allelopathic activity of a plant and the allelochemicals contents (10,16,30,42). However, little work has been done in this regard. This study aimed to investigate the effects of leachates of different phenological stages of *Nicotiana plumbaginifolia* on *Helianthus annuus* L. (sunflower) as test plant, as it is common weed in sunflower fields. It is a day neutral weed, which grows round the year in crop fields and is tertiary genetic relative of tobacco.

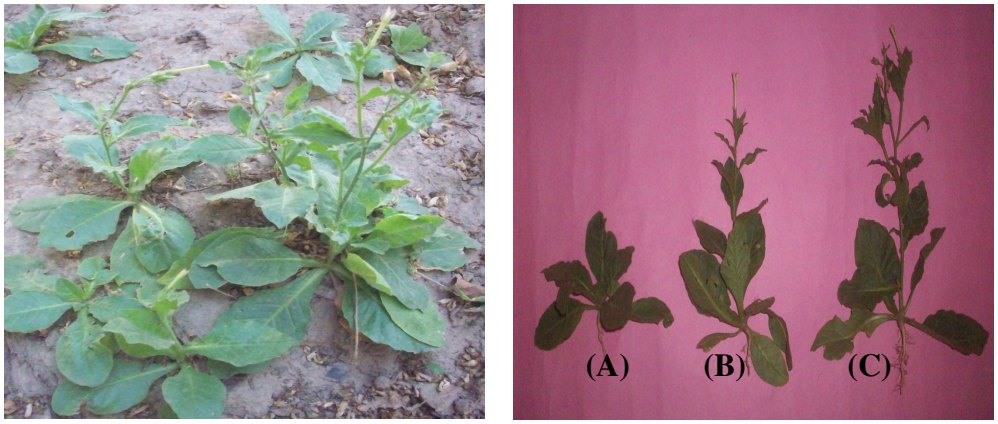
MATERIALS AND METHODS

Preparation of leachates

Plants of *Nicotiana plumbaginifolia* at 3-phenological stages (vegetative, flowering and fruiting stages) were collected separately in from the wild population found in the Roxburgh Botanical Garden, Department of Botany, University of Allahabad, Allahabad ($24^o47'$ and $50^o47'$ N latitude; $81^o91'$ and $82^o21'$E longitude; 78m above sea level). Plants of all 3-stages were collected in the same month as all stages of *N. plumbaginifolia* are found round the year (Photograph). These were collected in the month of September, 2007, two days before sowing of sunflower seeds. The uprooted plants were washed with tap water to remove the soil. The rain was simulated for 24 h, by a water dispersion unit, (submersible electric pump and rubber tube connected to a shower, which was placed over plant material with a stand). The pump was kept in the iron tub (50×50×25 cm) filled with 50 L distilled water. The donor plants were placed on netted cloth (made up of plastic fibers) which was tightly bound over the mouth of the tub. The sieves size in the net was 1.2× 1.2 mm. The plants were spread as thin layer over the net and the water was rained. It facilitated the free circulation of water (1:10 W/V). The leachate collected in the tub was taken out and filtered. The same practice was repeated to obtain the leachates of *Nicotiana* plants of all three stages. The mother leachates were raised to original volume by adding water. These were considered as leachates of 100% concentration and were kept in refrigerator at 8°C to avoid biodegradation. The osmolality of the leachates was measured with a vapour pressure osmometer (VAPRO 5520). It varied between 104-105 m mol kg^{-1} which did not cause any osmotic effect. The pH of leachates of different phenological stages was adjusted to 7.2. The leachates of 25, 50 and 75% concentrations were made by diluting the mother leachates with distilled water. These leachates were used to study their effects on germination and seedling growth (Experiment 1), pigment and protein contents, and activities of nitrate reductase (NR) and some antioxidants (Experiment 2).

Experiment 1: Petri sand culture

Healthy seeds of sunflower (*Helianthus annuus* cv. PAC-36) were surface sterilized in 0.001 M $HgCl_2$ and were washed with double distilled water thoroughly. The sterilized seeds were soaked in distilled water (control) and the respective leachate solutions of *N. plumbaginifolia* (25,50,75 and 100%) for 4 h. After that, 10 seeds of each treatments and control were placed in 9 cm dia Petri plates separately, filled with 25 g sterilized sand and irrigated with 10 mL of the respective solutions. Whole set up was kept in culture room (temperature: 28 ± 2^0C, humidity: 61±5 %). Germination and length of radicle and plumule were recorded at 24 h intervals.



Photoplate: *Nicotiana plumbaginifolia:* Three phenological stages (A) vegetative (B) flowering and (C) fruiting stage

Germination rate (GR) and absolute growth rate (AGR) were calculated as under:

$$GR = \sum \text{Number of seed germinated / day}$$
$$AGR = (h_2-h_1) / (t_2-t_1) \text{ cm/day},$$

Where, h_2 and h_1 are final and initial height of seedling; t_2 and t_1 are final and initial days.

Experiment 2: Soil Culture

Twenty five sterilized seeds of uniform weight were sown in each experimental tray (length: 30; width: 30; height: 10 cm) in 5 equidistant beds filled with 6 kg homogeneous soil. The row to row and plant to plant spacing was 5 cm. The experiment was conducted in culture room (temperature: 28±2°C; photoperiod: 18h; humidity: 61±5% and photon flux density: 240µ mol m^{-2} s^{-1}). Treatments were replicated thrice in randomized block design. The beds were irrigated with 200 ml of 25, 50, 75 and 100% concentration of leachate as per treatments. The beds irrigated with distilled water were taken as control. First fully expanded leaves from 15 d old seedlings were taken for biochemical analyses.

Measurement of pigments and protein contents

The pigments (leaf chlorophyll a, chlorophyll b and carotenoids), were extracted with 80% acetone and quantified following Lichtenthaler (24). Protein content was determined as per method of Lowry *et al.* (26). The amount of proteins was calculated with reference to standard curve obtained from bovine serum albumin.

Extraction and assay of enzymes

(i). **Nitrate reductase (NR- EC 1.7.99.4)** : Its activity was assayed by modified procedure of Jaworski (21) 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. 0.4 ml aliquot 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 from $NaNO_2$ and expressed as µ mol NO_2 g^{-1} FW h^{-1}.

(ii). **Superoxide dismutase (SOD-EC 1.15.11)** : Its activity was determined by the nitroblue tetrazolium (NBT) phototochemical assay method following Beyer and Fridovich (7). 0.2 g fresh leaf tissue was homogenized in 1% polyvinyl pyrrolidone (PVP) prepared in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged at 15000 g for 30 min at 4 °C. The reaction mixture contained 0.5 ml clear supernatant, 2 ml 0.15 mM ethylene di-aminetetra acetic acid (EDTA), 20 mM methionine, 0.12 mM NBT and 0.5 ml 11.96 µM riboflavin, 0.5 ml PVP and determined spectrophotometrically against blank at 560 nm. One unit of enzyme was defined as the amount of enzyme which caused 50% inhibition of NBT reduction.

(iii). **Catalase (CAT-EC 1.11.1.6)** : It was assayed according to the method of Sinha (36). 0.2 g of fresh leaf tissue was homogenized in 100 mM potassium phosphate buffer (pH 7.0) and centrifuged at 10,000 g for 30 min at 4°C. The reaction mixture contained 0.5 ml enzyme extract, 1.25 ml 0.2 M H_2O_2, 3.2 ml potassium phosphate buffer. After three minutes the reaction mixture was mixed with potassium dichromate acetic acid reagent. Absorbance was monitored at 570 nm and one unit of enzyme activity was defined as the amount which produced an increase of 0.1 OD per minute.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) by using SPSS (Ver.10; SPSS Inc., Chicago, IL, USA). Appropriate standard error of means (±SE) was calculated for presentation with tables and graphs. The treatment means were separated by Duncan's multiple range test (DMRT) at $P< 0.05$.

RESULTS AND DISCUSSION

Petri plate assay

Germination in sunflower initiated after 48 h and was recorded upto 5^{th} day when the germination was almost stabilized. Maximum germination was recorded in control which was decreased in different treatments. Germination was not significantly different ($P<0.05$) at 25% concentration of leachate from all stages, however higher concentration exhibited significantly different. Minimum germination was recorded in higher concentration of leachate of flowering stage with inhibition of 78.56%. Length of radicle and plumule was recorded up to 6^{th} day. Higher concentrations caused reduction in length of both radicle and plumule. Flowering stage was found to be more inhibitory in comparison to vegetative and fruiting stages.

Inhibited GR and AGR indicated the phytotoxic nature of *N. plumbaginifolia* leachate. Allelochemicals inhibited the physiological processes, viz. activity of hydrolyzing enzymes during the germination (38). An indirect relation between lower

Table 1. Effects of leachate of different phenological stages of *Nicotiana plumbaginifolia* on germination and seedling growth of sunflower in petri plate sand culture.

Leachate Conc. (%)	Germination		Seedling length (cm)		Absolute growth rate (AGR)
	Per cent	Rate	Radicle	Plumule	
Control	93.3 a	5.53 a	5.20 a	3.90 a	1.83 a
			Vegetative Phase		
25	93.3 a	5.50 a	5.20 a	3.80 ab	1.80 ab
50	76.7 c	4.53 bc	5.00 a	3.50 abc	1.70 abc
75	63.3 cd	3.83 cde	4.80 ab	3.40 bc	1.68 abc
100	30.0 e	1.70 f	4.30 cd	3.40 bc	1.66 abcd
			Flowering Phase		
25	83.3 ab	5.09 ab	3.90 de	2.90 d	1.41 cdef
50	66.7 cd	4.00 cd	3.80 e	2.60 de	1.37 def
75	30.0 e	1.78 f	3.80 e	2.40 ef	1.29 ef
100	20.01 e	1.32 f	3.50 e	2.07 f	1.15 f
			Fruiting Phase		
25	83.3 ab	5.00 ab	5.00 a	3.80 ab	1.59 abcde
50	76.7 bc	4.53 bc	5.00 a	3.60 abc	1.55 abcde
75	63.3 cd	3.67 de	4.80 ab	3.30 c	1.50 bcde
100	56.7 d	3.20	4.50 bc	3.40 bc	1.43 cdef

Mean values followed by the different letters within each column are significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3. Data were recorded on 2 to 5 days after sowing.

germination and inhibition due to allelochemicals may be the consequence of inhibition of water uptake (34). Phenolic compounds inhibit the activity of gibberellic acid (12), which regulates the *de novo* amylase production. Higher concentration of alkaloids inhibit the conversion of food reserves into soluble sugars (12), the substrate for respiration (Table.1)

Soil Culture

Allelopathins present in leachate significantly decreased the shoot and root lengths. However, the root was more susceptible to leachate. In the seedlings treated with the leachate obtained at flowering stage of *N. plumbaginifolia*, a decrease of 45.64% and 29.10% was recorded in root and shoot length, respectively. Leachate from flowering stage was more inhibitory followed by vegetative stage and was concentration dependent (Table 2).

Allelochemicals caused impairment of metabolic activities which resulted in decreased growth of seedlings. Allelochemicals cause premature lignification of root cells (11) and inhibit cell division and expansion (12). Consequently, root length was decreased which in turn contributed to a decrease in shoot length. Greater susceptibility of root to allelochemicals could be attributed to its direct physical contact with allelochemicals adsorbed on the surface of soil particles. Presence of more allelochemicals in the leachate obtained from flowering stage resulted in a greater inhibition as compared to that of vegetative and fruiting stage.

Pigments and protein content

Leachate significantly decreased the content of pigments. Maximum inhibition of 62.54% in the amount of total chlorophyll was recorded in the seedlings of sunflower

Table 2. Effect of leachate of different phenological stages of *Nicotiana plumbaginifolia* on seedling growth of sunflower in Pot culture.

Leachate Conc. (%)	Seedling growth	
	Root Length (cm)	Shoot Length (cm)
Control	10.10 a	11.20 a
	Vegetative stage	
25	9.67 a	10.71 a
50	9.01 b	9.38 bcd
75	8.81 bc	8.97 d
100	7.39 e	8.10 e
	Flowering stage	
25	8.37 cd	9.67 bc
50	7.96 de	9.11 cd
75	6.09 f	8.23 e
100	5.49 g	7.94 e
	Fruiting stage	
25	10.05 a	11.01 a
50	9.95 a	10.83 a
75	9.11 b	9.93 b
100	7.89 de	9.31 cd

Mean values followed by the different letters within each column are significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3. Sampling was done on 15 days after sowing.

treated with 100% concentration of leachate derived from flowering stage *Nicotiana plumbaginifolia.* It was followed by leachate of vegetative stage which caused maximum inhibition of 51.24% in its 100% concentration. Carotenoids also decreased in the treatments and followed the pattern of decrease in amount of total chlorophyll (Table 3).

Decrease in photosynthetic pigments due to allelochemicals was also recorded by many authors (4,40,41). The reduced content of chlorophyll in seedlings treated with leachate might be due to inhibition of chlorophyll synthesis, the stimulation of chlorophyll degradation or both (28). Results of concentration dependent decrease in chlorophyll content are in agreement with our previous work (2,36,39). Increased ratio of chlorophyll a/b indicated the more decrease in the content of chlorophyll b. The results showed maximum inhibition of 81.32% and 53.65% in chlorophyll b and chlorophyll a respectively. Increased chlorophyll a/b ratio cab be explain by the fact that first step of chlorophyll b degradation involves its conversion to chlorophyll a (14). Allelochemicals inhibit the activity of 4-hydroxyphenylpyruate dioxygenase, the key enzyme involved in biosynthesis of carotenoids (32).

Protein content decreased in all treatments and 100% concentration of leachate of flowering *Nicotiana* caused maximum inhibition (75.68%) followed by 100% concentration of leachate from vegetative stage (Fig. 1a). Decreased level of protein might be an adaptation to allelochemical stress. Protein degrades into amino acids such as proline which serves as osmolyte Leaf leachate of *Croton* increased the content of proline and asparagines in the leaves of *Parthenium* (35). Proline also serves as non-enzymatic antioxidant (20). Besides stimulation of degradation of protein, allelochemicals also inhibits the synthesis of protein (13,37). Thus decrease in protein content may be due to stimulation of degradation, inhibition of synthesis or both.

Table 3. Effects of leachate of different phenological stages of *Nicotiana plumbaginifolia* on pigments content of sunflower.

Leachate Conc. (%)	Pigments (mg/g FW)				
	Chlorophyll a	Chlorophyll b	Total chlorophyll	Chlorophyll a/b	Caroteniods
Control	1.92 a	0.91 a	2.83 a	2.11 f	0.74 a
			Vegetative stage		
25	1.76 ab	0.47 b	2.23 b	3.76 e	0.69 b
50	1.40 cd	0.35 c	1.75 de	4.01 de	0.61 c
75	1.36 de	0.30 cde	1.66 def	4.53 cde	0.50 e
100	1.18 e	0.20 fg	1.38 g	6.23 a	0.31 h
			Flowering stage		
25	1.34 de	0.31 cd	1.65 def	4.31 cde	0.55 d
50	1.27 de	0.28 de	1.55 efg	4.65 cde	0.37 g
75	1.21 de	0.24 ef	1.45 fg	5.06 bcd	0.31 h
100	0.89 f	0.17 g	1.06 h	5.23 abc	0.26 i
			Fruiting stage		
25	1.74 ab	0.48 b	2.22 b	3.65 e	0.65 bc
50	1.70 b	0.42 b	2.12 b	4.05 de	0.42 f
75	1.69 b	0.35 c	2.04 bc	4.83 bcde	0.38 fg
100	1.57 bc	0.27 de	1.84 cd	5.81 ab	0.32 h

Mean values followed by the different letters within each column are significantly different at 0.05 (ANOVA and Duncan's multiple range test) n=3.

Activities of nitrate reductase and antioxidant enzymes

Nitrate reductase is important for nitrogen metabolism and its activity has been shown to be directly correlated with crop yield (22). Nitrate reductase activity (NRA) was significantly decreased in the seedlings treated with leachate of different phenological stages of *Nicotiana plumbaginifolia.* However, the leachate from flowering *Nicotiana* caused more inhibition followed by vegetative and fruiting stage. 100% concentration of leachate from flowering stage caused maximum reduction (63.94%) in NRA (Fig. 1b). Reduced NRA under the influence of leachate was also reported by Bagawathy *et al* (4) and Tripathi *et al* (40). Allelochemicals influence the ion uptake. Allelochemicals decreased the absorption of nitrate by the roots and its transport from roots to leaves (1). This resulted in decreased foliar nitrate which in turn caused reduced NRA. The NRA is also very sensitive to plant water status (15). Allelochemicals alter the plant water relationship (6). So the decreased plant water status due to allelochemicals present in leachate might be responsible for such reduction in NRA.

The activity of antioxidants in the sunflower seedlings was variously influenced by the allelochemicals present in aqueous leachate of *N. plumbaginifolia*. Leachate stimulated the activity of SOD in all the treatments except the seedlings treated with 100% concentration of leachate collected at flowering stage (Fig 2a). CAT activity also increased in the seedlings treated with different concentration of leachate. However, 100% concentration of leachate from flowering and vegetative stage reduced the CAT activity by 33.33% and 24.42% respectively (Fig 2b). Allelochemicals caused oxidative stress and altered the activity of different antioxidant enzymes (33,34). Superoxide radical ($O_2^{\cdot-}$) is

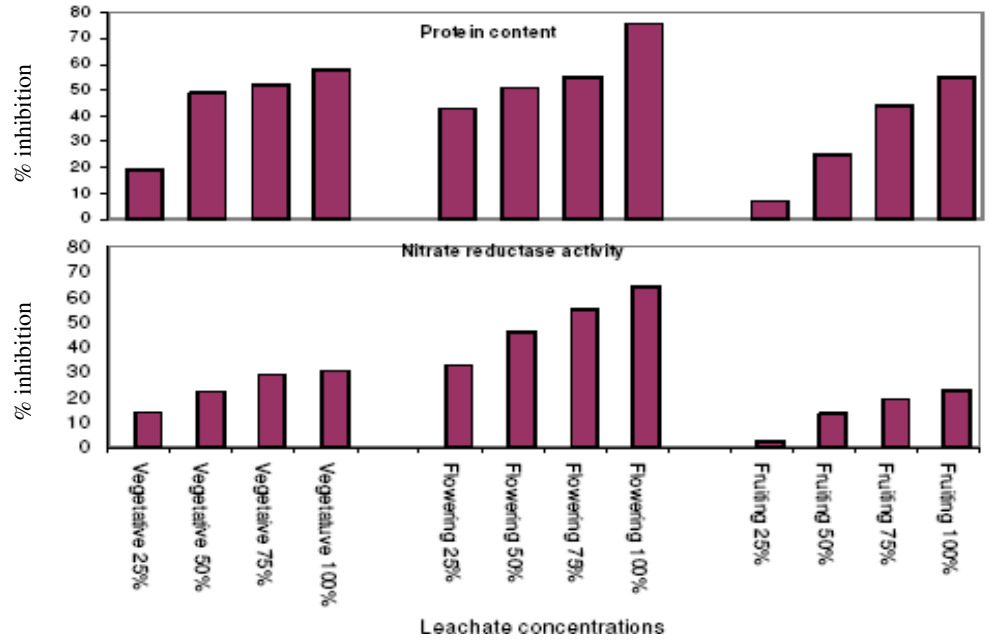


Figure 1. Effect of leachate of different phenological stages of *Nicotiana plumbaginifolia* on sunflower.

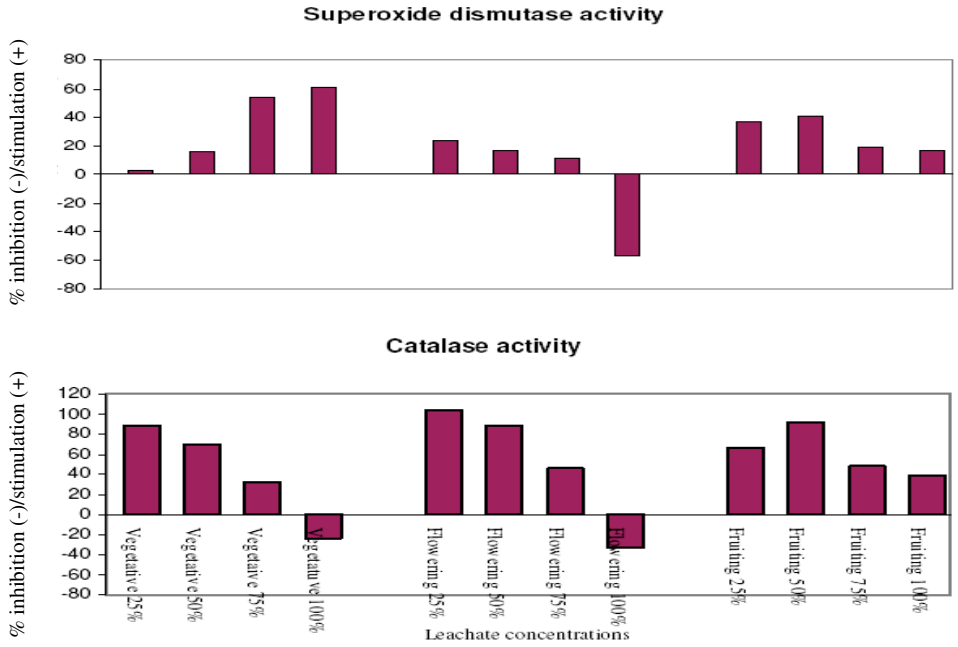


Figure 2. Effect of leachate of different phenological stages of *Nicotiana plumbaginifolia* on sunflower.

scavenged by the superoxide dismutase (SOD) which catalyses the dismutation of $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) and constitute the first line of defence against oxidative stress (18). But as H_2O_2 is itself toxic, it is further degraded by a wide range of enzymes, the most important being catalase (CAT) (8). Similar to our results allelochemicals present in leaf extract of sunflower caused oxidative stress in mustard seedlings through generation of ROS and enhanced the activities of SOD (25) and CAT (9). CAT activity decreased with increase in concentration of leachate. Decrease in CAT activity was also recorded by Rani (31) in case of *Ricinus communis* treated with higher concentration of *Piper betle* extract. Decrease in SOD and CAT activities caused by higher concentration indicated the possible failure of antioxidant defense system.

The physiological parameters previously depicted, clearly indicated the phytotoxic nature of the aqueous leachate of *Nicotiana plumbaginifolia* and its dependency on the phenological stages of growth. Flowering stage was the most allelopathic. The greater phytotoxicity of the leachate obtained at flowering stage simply reflects the greater concentration of inhibitory allelochemicals. The concentration of allelochemicals varies with age and plant organ (13). Phenolic profile of pepper plants show variation in relation to variety and phenological stages of plant growth (19). Amount of an indole alkaloid, gramine in barley decreased with plant age (45). In our experiment, flowering stage was found to be the most allelopathic owing to enhanced concentration of inhibitory allelochemicals at flowering stage. Our results are in agreement with Woodhead (43) who reported that phenolic acid concentration of sorghum leaves decreased with age of plants but increased again in the flowering stage. Similarily Baležentiene and Sampietro (5) reported that fodder galega (*Galega orientalis*) shoot have the lowest concentration of allelochemicals in early as well as in the last growth stages. Greater phytotoxicity at flowering stage may also be attributed to accumulation of a higher amount of allelochemicals in flowers. In *Parthenium hysterophorus* inhibitory chemicals are present in higher concentrations in leaves and flowers (23).

The present study revealed that the allelopathic potential of *Nicotiana plumbaginifolia* depends on its phenological stages and the flowering stage was the most allelopathic.

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