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Allelopathic impact of bark leachate of *Acacia nilotica* (L.) Delile on seed germination and enzymatic activities of *Phaseolus vulgaris* L.

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ABSTRACT

Agro-forestry programme in India has encouraged the farmers to grow different species of *Acacia, Eucalyptus, Albizia* and *Prosposis* in and around their crop fields to fulfil their demands. But recently attention has been focused on allelopathic effect of such trees on germination, growth and yield of the concerned crop. In order to investigate the allelopathic effect of aqueous bark leachate of *Acacia nilotica* on germination and activities of different enzymes concerned with germination of *Phaseolus vulgaris,* different concentrations of bark leachate (2-10%) were taken. Pure line seeds of *P. vulgaris* were germinated in plastic trays (containing cottons) in B.O.D. incubation. Experiments were conducted during 24-144 h for seed germination and 48-144 h for a-amylase and protease activities. Increased concentration of aqueous bark-leachate exhibited a negative correlation with seed germination and biochemical content of germinating seeds. However, a-amylase and protease activities were shown a positive correlation. The bark-leachate of *Acacia nilotica* contains allelochemicals which might have inhibited the seed germination and reduced the biochemical (carbohydrate and protein) content, whereas a-amylase and protease activities of germinating seeds were increased.

1. Introduction

Till 1970, little information was available on allelopathy in agro forestry system but thereafter, a lot of research works have been carried out. *Acacia* species affect crop growth by competing for various environmental resources than their little interference with the establishment and growth of the adjoining crop plants (Kohli *et al.,* 2006). Different species of *Acacia* release ferulic, vanillic, caffeic, gallic, m-hydroxybenzoic, and m-hydroxyphenyl acetic acids, tannins, flavonoides and gums, including phenolics in their litter. These chemicals may act in many biological processes, such as to suppress the mineral uptake by plants, inhibit cell elongation and cell division, as well as retard photosynthesis, respiration and enzymatic activities, resulting in the retardation of plant growth (Seigler, 2003; El-Khawas *et al.*, 2005). Carbelleria and Reigosa (1999) have demonstrated that the leachate from *Acacia delbata* showed strong inhibitory effects on the germination and growth of *Lactuca sativa*. Allelopathy has been implicated (Rafiqual-Hoque *et al.,* 2003; El-Khawas and Shehata, 2005). It has been observed that water extracts contains a wide range of chemicals of different *Acacia* species which inhibit germination, root and shoot length and also dry weight of different crops (AI-Wakeel *et al.* 2007; Lorenzo *et al*., 2008).

Phytochemical screening of the stem-bark of *Acacia nilotica* revealed that the plant contains terpenoids, alkaloids, soponins and glycosides (Banso, 2009). Plants, which are rich in alkaloids, tannins and glycosides, have shown to possess antimicrobial activity against a number of organisms. 2-Benzoxazolinone (BOA), a most potent allelochemicals of rye, suppresses the plants growth including crops and weeds (Burgos and Talbent, 2000; Beiz and Hunkle, 2004). The action of allelochemicals in target plant is diverse and affects a large number of biochemical reactions resulting in modifications of different processes. Allelopathic compounds may induce a secondary oxidative stress manifested as enlarged production of reactive oxygen

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species (ROS). ROS are known to act as signalling molecules, regulating plant response to biotic and abiotic stresses. Plant growth and development response to stress is controlled by phytohormones. Hormonal signalling transduction depends on ROS production. Hence, a study was therefore planned to assess the allelochemical effects of bark-leachate of *Acacia nilotica* on seed germination and enzymatic activities of (a-amylase and protease) and changes in carbohydrate and protein contents of germinating seeds.

2. Materials and methods

Freshly fallen bark of 10 years old *Acacia nilotica* tree grown in Berhampur University campus (19^0-60' N and 84^0-53'E) were collected at morning time. The barks were washed thoroughly with tap water and then with distilled water and thereafter dried in an incubator at 40±2^{0}C. The dried materials were chopped and then ground to make powder. Two hundred grams of each ground material were leached for 48 hours in one litre of distilled water and filtered separately (Padhy *et al,* 2000).These 20 % concentrated leachates were diluted to get desired concentrations (2-10%) for treatment.

Pure line seeds of *P. vulgaris* were procured from Regional Agricultural Research Station of Odisha University of Agriculture and Technology (O.U.A.T.), Berhampur located at Ratnapur. Healthy seeds of uniform colour, size, and shape were surface sterilised with 0.03% formalin solution for 10 min and then washed thoroughly with tap water followed by distilled water. Then seeds (@ 20 seeds / tray) were allowed to germinate in plastic trays (3 x 9 x 12 cm) having a thin bed of sterilized cotton as per the experimental schedule and containing various concentration of leachate. Incubation was done in dark in a B.O.D. incubator maintained at 30±1^{0}C. The germination (emergence of radicle) was observed at interval up to 6 days.

2.1. Extraction and assay of a-amylase

Germinated seeds from both control and treated sets were collected at random, soaked on blotting paper, weighed 1 g and then were ground with 2-3 ml of citrate buffer (pH 7.0) with a pinch of calcium chloride. The homogenates were centrifuged at 2000g for 15 minutes at 28±1^{0}C and the process was repeated thrice. The supernatants so collected were pooled together and made up to 10 ml with citrate buffer (Malik and Singh, 1980).

For assay of a-amylase activity, a reaction mixture was prepared by adding 1 ml of starch solution (0.15% in 0.04 M KH_2PO_4), 0.5 ml of enzyme extract and 3 ml of Iodine reagent (0.6% iodine in 6% KI solution), allowed to stand for 10 minutes at room temperature and absorbance of the reaction mixture was recorded at 620 nm with help of a spectrophotometer.. The enzymatic activity was expressed in mg of starch hydrolysed per 10 minute per gram fresh weight of seeds from the standard curve prepared with different concentration of starch (15-150 mg/100 ml distilled water).

2.2. Total carbohydrate

The germinated seeds (100 mg) collected from control and treated sets at random were homogenised with 80% ethanol in pre-chilled mortar and pestle and the homogenates were centrifuged at 3000g for 15 minutes at 28±1^{0}C. The supernatants were collected by repeating the procedure thrice. The pooled supernatants were concentrated into 1-2 ml in a water bath maintained at 50±1^{0}C. The slurry was diluted with distilled water to a definite volume and then used for estimation of carbohydrates spectrophotometrically by anthrone method as per Plummer (1979). D-Glucose was taken for preparation of standard curve.

2.3. Extraction and assay of protease

Germinating seeds were collected at random and soaked on blotting paper. One gram of seeds from each treatment were homogenized with 2-3 ml of 0.05 M Tris malate buffers (pH 7.0), equal volume of 40 mM cysteine hydrochloride and 4% sodium chloride solution were added and pH was adjusted to 7.0 with 0.1 N aqueous NaOH solution. The homogenate was centrifuged at 17000g for 20 minutes at 28 ±1°C. The process was repeated thrice and supernatants so collected were pooled together and the volume was made up to 10 ml with tris-malate buffer. Bovine serum albumin (BSA) was used as a substrate. Three millilitre of the assay mixture (1.5 ml of 0.05 M tris-malate buffers (pH 7.0), 0.5 ml of 2% BSA and 1 ml of enzyme extract) were incubated for 2 hours at 40±1^{0}C and the reaction was stopped by adding 1 ml of 20% trichloroacetic acid (TCA). A zero time control was run at the same time with adding enzyme solution immediately before addition of TCA. Then the assay mixture was kept overnight in a refrigerator and centrifuged at 15000g at 4±1^{0}C for 15 minutes. The process was repeated thrice and the supernatants were pooled together and adjusted to pH 5.0 with 0.1 N NaOH. Then 0.5 ml of supernatant was added to 1.0 ml of Ninhydrin reagents and heated for 10 min at 100^{0}C. Four ml of distilled water were added to each sample and the absorbance was measured at 570 nm (Moore and Stein, 1948). Protease activity was expressed in mMole of NH_2/hours.g.fr. wt. of seed.

2.4. Estimation of protein

The pellets left over after extraction of carbohydrates from germinated seed were suspended in 5% (w/v)

Trichloroacetic acid (TCA) at 0°C for 15 minutes and centrifuged at 5000g for 20 minutes. The process was repeated twice and supernatants were discarded. Then 2 ml of 1N NaOH was added to pellets present in centrifuged tubes. The tubes were left as such at room temperature for 30 minutes, then kept in a boiling water bath for 15 minutes, cooled and centrifuged at 5000g for 15 minutes to room temperature. The insoluble protein present in supernatant was estimated by folin-phenol method as described by Lowry *et al.* (1951) taking bovine serum albumin as standard.

3. Result and discussion

3.1. Seed germination

The aqueous bark - leachate of *Acacia nilotica* significantly reduced the germination in green gram. All concentrations of test leachate caused reduction of seed germination and the rate of reduction increased with the concentration of leachate (Table 1). These results are in agreement with those obtained by Duhan and Lakshminarayan (1995), who found that the growth of *Cyamposis tetragonal* growing at distance of 1-2 m from the tree *Acacia nilotica* was inhibited. Inhibition of seed germination with increased leachate concentration was observed by different authors on the other plants viz. maize and kidney bean by leave extract of *Eucalyptus globules* (El-Khawas and Shehata, 2005), mung bean by leaf extract of *Lantana camara* (Maiti *et al*., 2010) and wheat by Eucalyptus (Patil *et al*., 2002). Many other species of genus Acacia, like *A. delbata* (Carbelleria and Reigosa, 1999; Lorenzo *et al*., 2008), *A. confusa* (Chou *et al*., 1998). *A. auriculaeformis*, *A.cunn* (Rafiqual-Hoque *et al*., 2003, Dash 2012) and *A. nilotica* (Al-Wakeel *et al*., 2007) are known to exhibit allelopathic activity.

The effects of allelochemicals have been studied mostly on seed germinations and the mechanisms of inhibition are mostly by disruption of mitochondrial respiration (Abrahim *et al.*, 2000) and interference in normal cell division (Padhy *et al.*, 2005). Effects of allelochemicals on seed germination appear to be mediated through a disruption of normal cellular metabolism rather through damage of organelles. Reserve mobilisation; a process which usually takes place rapidly during early stage of seed germination seems to be delayed under allelopathic stress.

3.2. a-amylase and carbohydrate

The amount of total carbohydrate content in germinating seeds considerably decreased with increase of leachate concentration (Fig.1a). Similarly the a-amylase activity also gradually increased (Fig.1b). Maiti *et al.* (2001) reported that the inhibition of germination behaviour was associated with deceased level of carbohydrate and increase of a-amylase activity in mung bean seeds affected by leaf extracts of *Lantana camara*. The present findings corroborate with report of several workers on the other plants (Maiti *et al.,* 2008; 2010; Das *et al.,* 2012; Dash, 2012).

3.3. Protease and protein

All the concentrations of the leachate considerably exhibited reduction in protein content and increase in the protease activity in germinating seeds (Fig 2 a & b). The present finding corroborate with the findings of different workers in other plants influenced by phytochemicals (Maiti *et al.,* 2008; 2010; Dash, 2012). However, the protease activity in plants influenced by allelochemicals were decreased with increase of leachate concentration reported by other workers (Kohli and Pariana, 1992; Pawar and Chavas, 2004; Gantayat *et al*., 2006).

There is a reduction of protein and carbohydrate content in treated seeds, which possibly played a significant role in the deterioration of the germinating seeds. The activity of a-amylase and protease significantly increased in pre-treated seeds samples than control. These enzymes play a vital role during germination and growth (Maiti *et al.,* 2008). a-amylase activity regulates starch breakdown, necessary

Table 1
Impact of different concentrations of aqueous-bark-leachate of *Acacia nilotica* on germination (%) of green gram seeds at different days after soaking (DAS). Values are means of 5 replicates ± S. E. M.

DAS	Leachate concentration (%)					
	0	2	4	6	8	10
2	20.28 ± 0.53	18.26 ±0.49	13.44±0.33	9.82±0.22	3.20±0.36	0
3	43.82 ±0.29	37.24 ±0.29	31.86±0.27	28.28±0.56	19.22±0.43	11.46±0.26
4	67.44 ±0.24	61.28 ±0.32	55.24±0.21	46.62±0.22	35.26±0.27	25.24±0.24
5	87.28±0.36	79.44±0.41	71.26±0.53	57.24±0.39	43.82±0.21	35.26±0.46
6	95.44±0.42	87.26±0.40	79.24±0.31	65.62±0.33	51.62±0.34	43.28±0.32

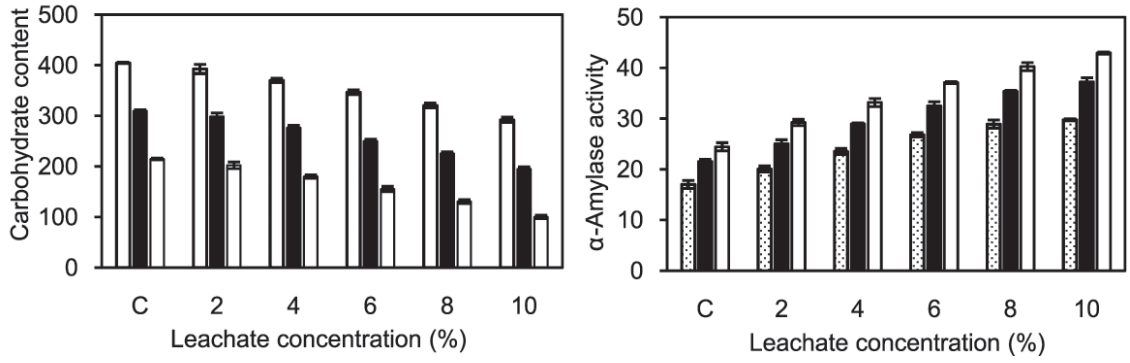

Fig. 1. Changes in total (A) carbohydrate content (mg/g fr. wt) and (B) α-Amylase activity (mg of starch utilised/10 min/g fr. wt.) of germinating green gram seeds at different days after soaking (DAS) influenced by varying concentrations of aqueous-bark-leachate of *Acacia nilotica.* Columns: 2 DAS (variegated); 4 DAS (full); 6 DAS (empty).

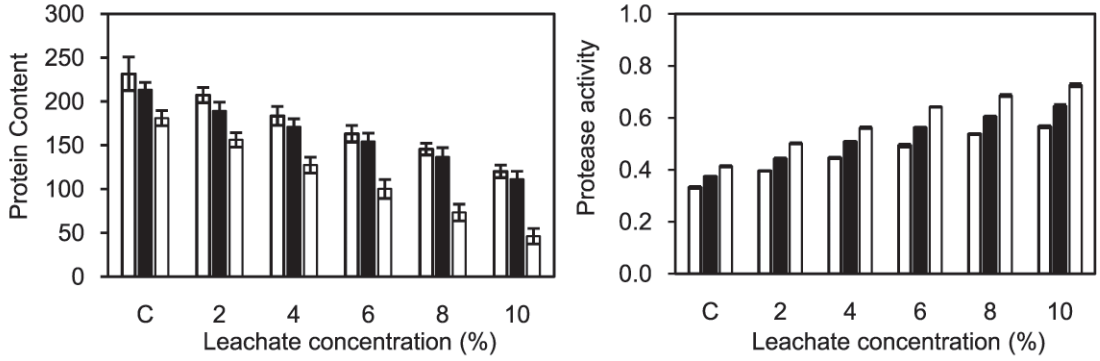

Fig. 2. Changes in (A) protein content (mg / g. fr. wt.) and (B) protease activity (m mole of NH_2 released/g fr. wt.) of germinating green gram seeds at different days after soaking (DAS) influenced by varying concentrations of aqueous-bark-leachate of *Acacia nilotica.* Columns are as for figure 1.

for supplying substrate to respiratory metabolism. Increased activities of a-amylase suggest more utilisation of sugars to meet the increased energy demands of tissues in response to allelochemicals induced stress (Daizy *et al.*, 2006).

Seed germination is a very sophisticated process which required the concerted action and interaction between diverse phytohormones. Seed dormancy and germination are complex traits that are regulated by the antagonistic action of the phytohrmones abscisic acid (ABA) and gibberellins. ABA is considered as a major stress hormone and its accumulation is connected to dehydration related process in plants (Houser *et al.*, 2011). ABA functions as a positive regulator of physiological dormancy while GA and Ethylene negatively regulated dormancy and promote seed germination. Allelochecals might have inhibited the seed germination by suppressing the synthesis of gibberellins and indol acetic acid. Allelopathic compounds may reduce a secondary oxidative stress manifested as enlarged production of reactive oxygen species (ROS). ROS are known to act as signalling molecules, regulating plant response to biotic and abiotic stresses. ROS have been implicated as second messengers in plant hormones response.

It is concluded that the allelochemicals present in bark leachate of *Acacia nilotica* exhibited a negative correlation with biomolecular content (carbohydrate and protein) and increased leachate concentration. Increased a-amylase and protease activities were responsible for breaking down of more carbohydrate and protein to simple form which may serve as respiratory substrate and release large amount of energy required to facing the allelochemical stresses. Allelochemical stress induced redox transformation that ultimately result in the formation of ROS and they imbalance different growth hormones, secondary metabolites and signal molecules which might have reduced the seed germination. Further the studies can be extended regarding the efficacy of these bark leachate under field condition, isolation and identification of allelochemicals responsible for germination, biomolecular content and enzymatic activities in the crop plants.

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