CRITICAL ANALYSIS OF ALLELOPATHIC STATUS OF *Eucalyptus globulus* Labill. THROUGH PHYSIOBIOCHEMICAL AND CYTOLOGICAL APPROACHES

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Abstract: Allelopathic potential of *Eucalyptus globulus* was evaluated using its fresh leaf extracts and dry leaf leachates of young, mature and old leaves using black gram (*Vigna radiata*) seeds as bioassay material. Pretreatment of black gram seeds with different concentrations of aqueous leaf extracts and leaf leachates of the *Eucalyptus* for 8 hours reduced percentage and speed of seed germination with concomitant enhancement of the time required for 50% germination (T_{50}) of *Vigna* seeds. Growth of seedlings, measured in terms of root and shoot length, fresh and dry weight of seedlings, raised from the seeds pretreated with the leaf extracts and leaf leachates were significantly impaired.

The seed pretreating agents enhanced deleterious leaching of soluble carbohydrates and free amino acids from the seeds with concomitant reduction of protein and nucleic acids as well as activities of catalase and dehydrogenase. Treatment-induced impairment of chromosomal behaviour during mitosis and reduction of Mitotic Index (MI) were clearly observed.

A conclusion is made that the *Eucalyptus globulus* possesses allelopathic property.

***Index Terms*: Allelopathy, *Eucalyptus globulus, Vigna radiata,* seed germination behaviour, chromosomal behaviour, mitotic index.**

I. INTRODUCTION:

Allelopathy refers to either beneficial or harmful effect of one plant upon another, both crop and weed species, by the release of chemicals (allelochemicals) from plant parts by leaching, root exudation, volatilization, residue decomposition and other processes in both natural and agricultural systems. Whole body of the allelopathic plant or different parts including flowers, stems, barks, roots, leaves, leaf litter and leaf mulch, soil and soil leachates and the allelochemicals or their derivative compounds can have the allelopathic property. Inhibition of allelopathy is complex phenomenon which can involve the interaction of different classes of chemicals like phenolic compounds, alkaloids, flavonoids, terpenoids, steroids, carbohydrates, amino acids etc. The mixture of allelochemicals sometime produces greater effect than an individual compound alone. Allelopathy is also an expression of the ecological phenomenon which is a normal constituent of the environment of the terrestrial plants (Dutta and Sinha-Roy, 1974[8]; Vivanco *et al*, 2004[27]). In 1996, The International Allelopathy Society (IAS) put forward a definition of 'Allelopathy' in accordance with Rice (1984[20]), but effects of biochemical compounds involved in plant-plant interactions and the effects of allelopathic plants are discussed in a broader perspective than strictly related to plant-plant interactions. The IAS defined the allelopathy as: "Any process involving secondary metabolites produced by plants, micro-organisms, viruses, fungi that influence the growth and development of agricultural and biochemical systems (including animals), including positive and negative effects" (Torres *et al*, 1996). Allelopathy research has been conducted for several decades, but much knowledge knowledge is yet to be achieved in this field (Thorpe *et al*, 2009[24]). An improvement in crop cultivars is the only area that has not been exploited to any great extent as a weed management strategy (Callaway and Vivanco, 2007[5]). The possibility of incorporating allelopathic traits into improved rice cultivars, which would reduce the need for applying herbicides to the crop, is worth exploring. Of course, thus far, no commercial cultivars carrying allelopathic properties have been developed (Duke *et al*., 2002[9]).

The present study is an attempt to assess the allelopathic potential of a fast growing, exotic tree *Eucalyptus globulus* Labill. (Family- Myrtaceae). This species has been selected in view of the fact that many exotic displace the local biodiversity through their harmful effects including allelopathy (Bhakat, 2006[2]; Bhattacharjee, 2003[3]; Nayek *et al.*, 2002[18]; 2012[19]). Allelopathic efficacy was analysed by using black gram (*Vigna radiata*) seeds as a reliable bioassay material. In fact, allelopathic action of any plant or plant part affects germination behaviour, metabolism as well as growth and development of seeds and seedlings which in turn may discourage a species from thriving, thus influencing the whole community structure (Ghosh and Dutta, 1989[11]). The potential status of Vigna seeds were evaluated as a result of allelopathic treatment with donor plant under laboratory and field conditions.

II. MATERIALS AND METHODS:

All the experiments of the present study were carried out with the fully viable healthy seeds of black gram (*Vigna radiata*) procured from the Seeds Corporation of India, Midnapore District Office, West Bengal, India. The exotic species was taken into consideration for Allelopathic studies is *Eucalyptus globulus* as seeds hardly germinate under its canopy.

Fresh and healthy leaves of three types *viz*. young, mature and old were collected from actively growing populations of *Eucalyptus globulus* (Family Myrtaceae) in Vidyasagar University Campus Midnapore, West Bengal, during the period of investigation. The leaf samples were collected from the plants throughout its life cycle and washed separately with distilled water to remove the adherent dust particles.

To prepare leaf extracts fresh and healthy young leaves (100g) of *Eucalyptus* were thoroughly homogenized using 250 ml double distilled water. The homogenate was strained using a fine cloth and the filtrate was stirred manually for five min and subsequently centrifuged at 5000 *g* for 15 min. Then the sample was again filtered with the help of Whatman No.1 filter paper. The volume of the filtered solution was then made up to 500 ml using double distilled water and this was considered as 1:5 (W/V) proportion stock solution of the aqueous leaf extract. Thus, the concentration grade of 1:5 w/v of *Eucalyptus* of young and old leaf extract was used as the test sample for allopathic studies.

To prepare leaf leachates 100g sun-dried young leaf samples of each *Eucalyptus* were kept immersed in 250 ml double distilled water in 1000ml beaker and kept at room temperature (28±2°C) for 48 h. Then it was stirred manually for five min and filtered through Whatman No.1 filter paper to prepare aqueous leachate which was decanted in a separate beaker. The total volume of the leachate was then made up to 500 ml using double distilled water and it was taken as the 1:5 (w/v) proportion stock solution. Thus, the concentration grade of 1:5 w/v of *Eucalyptus* young and old leaf leachate was used as the test samples for allelopathic studies. Fully viable *Vigna radiata* seeds in twenty four lots of 25 g each were surface sterilized with 0.1% $HgCl_2$ solution for 90 seconds. The seed lots were then separately presoaked in the three types of leaf leachates and leaf extracts of *Eucalyptus* for 8 h and then allowed the seeds to dry back to original seed moisture level. Thus, the seeds were considered for biochemical tests.

Fully viable black gram seeds were taken and immersed in a beaker half filled with double distilled water and kept in a well ventilated place at room temperature. After sprouting of the plumule, seeds were transferred to wetted sand – saw dust mixture (1:1) and kept at room temperature (30 ± 2 °C). After 2 days, primary roots with 1-1.5 mm long were pulled out from the sand saw dust mixture carefully and washed thoroughly with distilled water. The roots were immersed within leaf extracts and leachates of *Eucalyptus* of different types carefully. The set up were kept in a well ventilated lighted place at room temperature (30 ± 2 °C) for 18 h. The roots were then pulled out from the sand solution mixture and rinsed thoroughly with distilled water. Root tips were cut at 2-3 mm of length and fixed for overnight in acetic acid and ethanol mixture (1:3) and were stored in 70% alcohol for subsequent use. Then root tip squashes were made by the haematoxylin technique.

To analyze the speed of germination, the individual seed lots in each groups of 100 seeds of each treatment were transferred separately to Petri dishes (9 cm) containing filter paper moistened with 10 ml double distilled water. Data were recorded at an interval of 24 h in laboratory up to 168 h of seed soaking in distilled water. Germination data were recorded following the rules of International Seed Testing Association (ISTA, 1976). The time required for 50% germination of seeds (T_{50}) was determined following the method described by Coolbear *et al*. (1984). Percentage germination was recorded by analyzing speed of germination of individual seed lots of each treatment after 7 days of seed soaking following the values of International Seed Testing Association (ISTA, 1976).

Growth of seedlings were recorded in terms of root length, shoot length, fresh weight and dry weight from 20 uniformly growing (28 days old) seedling raised from the treated seeds in the field.

Free amino acid levels from the seed leachates of each treatment were analyzed after immersing 10 seed sample of black gram in 10 ml distilled water for 24 h. The leachates were carefully separated from the seeds in separate test tubes. From the leachate stock, free amino acid level was quantified following the method of Moore and Stein (1948[17]) modified by Bhattacharjee (1984).

Soluble carbohydrate from seed leachate, sampling procedure was the same as done in case of leachable free amino acids, and from the same leachate stock. Soluble carbohydrate level was determined following the method of Mc Cready *et al* (1950[14]) after simple modifications.

Extraction of nucleic acids (DNA & RNA) were done from seed kernel following the method described by Biswas and Chowdhuri (1978) and estimated as per the method of Cherry (1962), modified by Choudhuri and Chatterjee (1970).

The activity of total dehydrogenase of intact seeds was analyzed by the reception of tetrazolium chloride according to the method of Rudrapal & Basu (1979[21]). The enzyme activity was determined followed the method of Snell and Snell (1971) modified by Biswas & Choudhuri (1978).

III. CODES USED FOR THE EXPERIMENT:

CON/Control = Seeds treated with double distilled water.
E1 = Seeds treated with Eucalyptus Young Leaf Leachate 1:5 (w/v).
E2 = Seeds treated with Eucalyptus Mature Leaf Leachate 1:5 (w/v).
E3 = Seeds treated with Eucalyptus Old Leaf Leachate 1:5 (w/v).
E4 = Seeds treated with Eucalyptus Young Leaf Extract 1:5 (w/v).
E5 = Seeds treated with Eucalyptus Mature Leaf Extract 1:5 (w/v).
E6 = Seeds treated with Eucalyptus Old Leaf Extract 1:5 (w/v).

IV. RESULT AND DISCUSSION:

The treatment induced inhibitory effects on seed germination were associated with reduction of percentage germination and deleterious effect of T_{50} (Table - 1). Concomitant of the chemical induced adverse effects on seed germination was associated with the slowing down of the speed of germination (Table – 2) after treatment. Seed pretreatment with the leaf and leaf extracts significantly enhanced leaching of soluble carbohydrates and free amino acids (Table – 5) irrespective of seed samples.

The above mentioned physiological changes were associated with the treatment-induced changes of a number of biochemical parameters analysed in seed kernels. The treatments enhanced the levels of amino acids, soluble carbohydrates, along with concomitant reduction of the levels of proteins, nucleic acids (DNA and RNA) (Table-6), as well as activities of dehydrogenase and catalase (Table-7).

There are reports in the literature that plants having allelopathic potential can reduce seed germinability, speed of germination along with deranging of seed membranes causing leaky membrane structure (Maiti, 2007[16]; Bhakat and Nayek, 2001[4]; Bhattacharjee *et al*, 2003[3]). Again, some biochemical parameters are reported to be adversely affected as a result of seed pretreatment with plant extracts and leachates having allelopathic effect (Zang, 2010[30]; Datta and Chatterjee, 1980[7]). My results are in conformity with the reported results. In fact, damage of seed membrane by putative allelochemicals present in leaf extracts and leaf leachates causes the seed membranes leaky/porous which consequently enhances membranes permeability and thereby, increasing the levels of soluble substances. Reduction of macromolecules like protein, nucleic acids (DNA and RNA) may be due to the reduced biosynthesis of these substances and/or due to the activities of the enzymes like protease, DNase, RNase by inhibitory effects of the allelochemicals. Subdued activities of dehydrogenase and catalase might be attributed to the deleterious effect of the allelochemicals present in the extracts and leachates of the plant samples.

Significant decline in the mitotic index of treated root tip cells of *Vigna radiata* undoubtedly revealed that the inhibitory effect of leaf extracts and leaf leachates of *Eucalyptus* on cell division (Tables 8 and 9). The inhibitory effect was marked in E3 and in on the cells of *Vigna radiata* as compared to control sample. The curious feature of the mitotic index curve is that retaining their individual difference between ages of leaves and/or extracts and leachates as compared to the control. The inhibitory effects indicate the action of leaf extracts and leaf leachates on metabolism of interphase nucleus. Decrease in the mitotic index indicates the cytotoxic effect (Ukaebu and Odeigah, 2009[26]). Reduction in number of dividing cells in the root meristem shows the antimitotic effect of the substance (Ali and Celik, 2007[1]). Choudhury and Sajid (1984[6]) reported that the decrease in mitotic index of pea root treated with highest concentration and longer duration might be attributed to toxicity of the fungicide which could not permit the meristematic cells to enter into division. In this investigation, the percentage germination (Table 1) was decreased along with slowing down the speed of germination (Table 2) and decrease of dehydrogenase as well as catalase activities (Tables 7) in the treated samples. The numbers of dividing cells were also reduced in samples treated with leaf extracts and leaf leachates of *Eucalyptus* (Tables 8 and 9). Thus, the results are indicative of the fact that the putative allelochemicals might be the causal agents for inhibition of the variables analyzed in this investigation (Maiti *et al*, 2008[15]).

The interesting feature is that the cytotoxicity was increased by young and mature leaf extract treatments. Higher percentage of abnormalities (Table 9) was recorded in cells treated with mature leaf extracts followed by young leaf extracts and old leaf leachate. The data presented here indicate that old and mature leaves of *Eucalyptus* have the potentiality of inducing a variety of abnormalities in the root tip cells of *Vigna*. Abnormalities manifested might be due to the action of some allelochemicals (Nayek, 2012[19]).

Table 1: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of percentage germination (%), time (h) for 50% germination (T_{50}) on black gram seeds.

Treatments	Germination	T_{50}
Control	99.67	16.5
E1	48.3	NA
E2	46.3	NA
E3	47.1	NA
E4	47.8	NA
E5	43.7	NA
E6	45.6	NA

NA: Non attainment of 50% germination

Table 2: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of speed of germination of black gram seeds.

Treatments	Germination at 12 h intervals									
	12	24	36	48	60	72	84	96	108	120
Control	27.6	58.3	67.3	75.1	83.5	90.4	93.3	95.6	97.1	99.6
E1	10.3	16.2	27.4	31.6	38.5	43.2	46.8	47.6	48.3	48.3
E2	9.4	12.3	23.1	28.1	35.8	40.8	43.7	46.1	46.3	46.3
E3	9.3	13.2	24.1	29.3	34.7	42.6	44.8	46.6	47.1	47.1
E4	10.1	12.3	22.4	30.1	35.6	43.5	46.2	46.8	47.3	47.8
E5	8.3	13.1	21.7	28.5	36.6	40.5	43.2	43.7	43.7	43.7

E6	9.2	14.2	24.3	30.1	37.0	41.6	44.8	45.3	45.6	45.6

Table 3: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of root length and shoot length of 28-days old plants raised from treated seeds.

Treatments	Root length (cm)	Shoot length (cm)
Control	14.85	33.26
E1	8.35	18.36
E2	6.81	15.71
E3	7.27	16.38
E4	7.88	17.43
E5	6.05	14.75
E6	6.40	15.28

Table 4: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of plant fresh weight and dry weight of 28-days old plants raised from treated seeds.

Treatments	Plant fresh weight (g)	Plant dry weight (g)
Control	41.73	6.12
E1	23.54	3.07
E2	16.15	2.11
E3	18.36	2.38
E4	20.21	2.64
E5	13.44	1.65
E6	14.95	1.88

Table 5: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of leaching of soluble carbohydrate and free amino acid levels of black gram seeds.

Treatments	Soluble carbohydrate (mg/g/10ml)	Amino acid (mg/g/10ml)
Control	11.72	1.74
E1	32.80	4.81
E2	40.10	6.10
E3	35.85	5.45
E4	43.14	5.12
E5	47.45	7.85
E6	43.60	6.48

Table 6: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of protein and changes of DNA and RNA contents in seed kernels black gram seeds.

Treatments	Protein (mg/g fr. wt.)	DNA (µg/g fr. wt.)	RNA (µg/g fr. wt.)
Control	41.80	75.36	870.56
E1	28.26	32.16	445.63
E2	24.15	29.53	361.21
E3	25.44	30.24	385.47
E4	27.00	31.67	410.13
E5	21.56	27.77	311.50
E6	23.75	28.18	337.20

Table 7: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on changes of dehydrogenase and catalase activities in seed kernels black gram seeds.

Treatments	Dehydrogenase (ΔOD/g/10ml)	Catalase (Unit/h/g fr. wt.)
Control	0.47	129.30
E1	0.18	77.82
E2	0.13	65.37
E3	0.16	71.33
E4	0.17	75.11
E5	0.09	55.89
E6	0.11	61.50

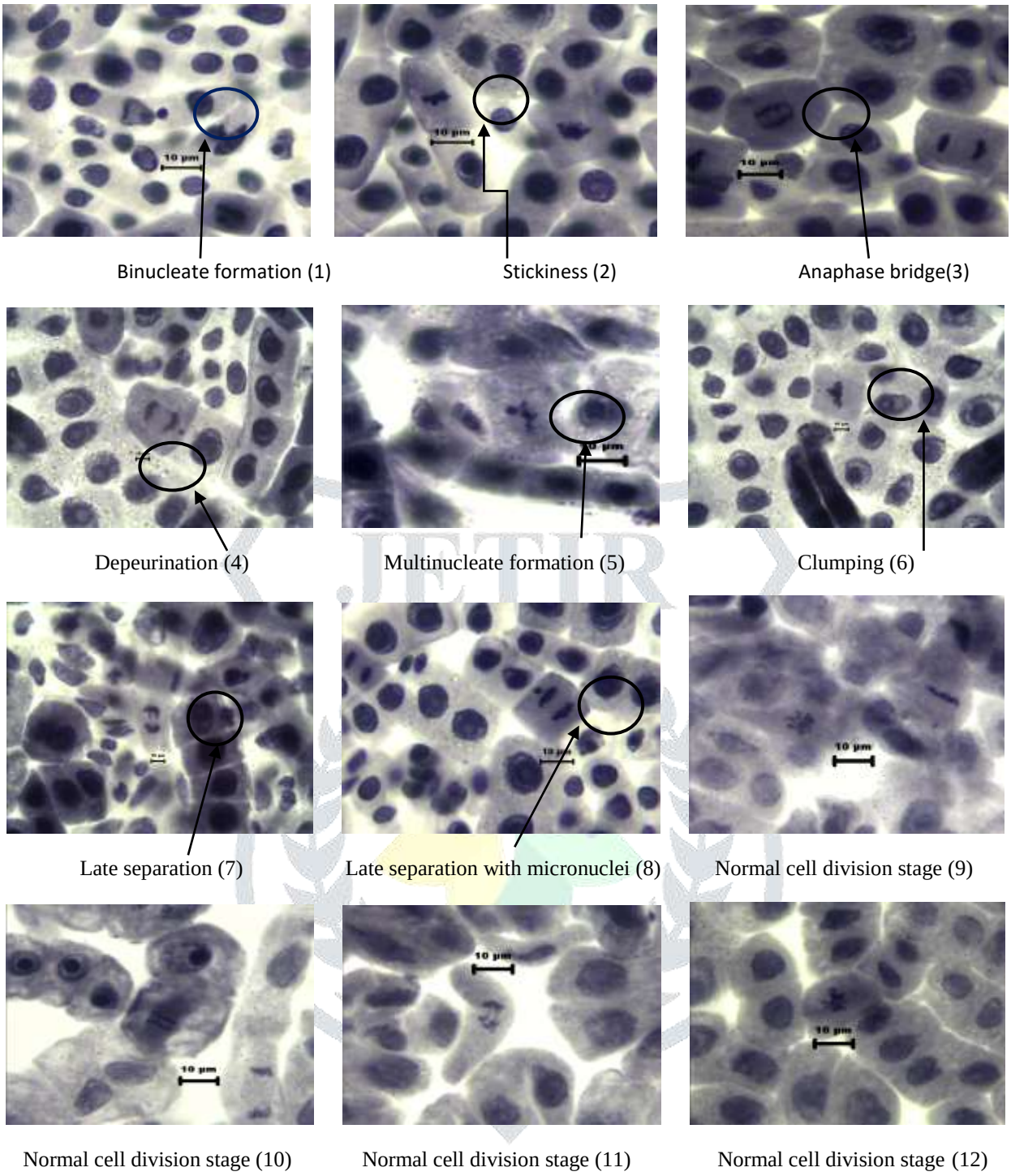
Table 8: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on mitotic behaviour of *Vigna radiata* root tips.

Treatments	Prophase cells	Metaphase cells	Anaphase cells	Telophase cells	Total deviding cells	Total cells counted	Mitotic Index (%)
Control	56	57	78	144	355	2976	11.92
E1	29	33	51	99	212	2786	7.60
E2	26	37	53	100	216	2863	7.54
E3	24	33	52	100	209	2832	7.37
E4	31	32	61	98	222	2851	7.78
E5	27	26	45	84	182	2837	6.41
E6	29	30	51	91	201	2844	7.06

Table 9: Effect of seed pretreatment with leaf extracts and leaf leachates of *Eucalyptus* on chromosomal aberrations of *Vigna radiata* root tips.

Treatments	Total dividing cell	Total cells counted	Late separation	Anaphase bridge	Clumping	Binucleate cells	Depeurination	Stickiness	Multinucleate cells	Late sepatration with micronucleus	Total abnormal cells counted	Abnormality index (%)
Control	355	2976	0	0	0	0	0	0	0	0	0	0
E1	212	2786	1	4	2	1	1	1	2	1	13	6.13
E2	216	2863	2	5	3	1	3	1	0	1	16	7.40
E3	209	2832	2	3	2	1	1	1	2	2	14	6.69
E4	222	2851	2	2	2	3	1	1	2	1	14	6.30
E5	182	2837	3	4	3	2	2	1	2	2	19	10.43
E6	201	2844	1	3	4	2	1	1	2	2	18	8.95

V. PHOTOGRAPHS:



Photographs showing the abnormal mitotic divisional stages in *Vigna* root-tip cells observed in treated (1-8) and control sample (9-12).

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