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Economics of Production, Collection and Utilization of Kewda (*Pandanus fascicularis* Lam.) Flowers in Ganjam District of Orissa, India

D.K. Mahapatra and S.B. Sahoo
Institute of Minerals and Materials Technology (CSIR), Bhubaneswar-751013

Introduction

"Kewda" botanically known as *Pandanus fascicularis* Lam. belongs to the family Pandanaceae and grows wild in India along the sea coast, river banks, etc.[1] The most luxuriant growth is found throughout the coasts of India, Myanmar, Java, Malaysia, China and Indonesia. In India it is widely distributed in the coastal districts of Orissa, Andhra Pradesh, Tamil Nadu, Kerala, Gujarat and some parts of Uttar Pradesh. Over 500 species of the genus of Pandanus are known, of which 36 species have been recorded in India.[1] The wild population of kewda grows over 5000 hectares of area and it is estimated that about 35 million (3500 tons) flowers are processed every season yielding essential oil worth Rs.400 million annually.[2] It is amazing that as on today, about 200 remote villages of Rangeilunda, Chatrapur, Ganjam and Chikiti blocks of Ganjam district of Orissa supply about 85-90% of country's Kewda essence. A huge number of flowers arise in the peak season i.e. in July-September. The total annual utilization of the flower in stills is only 20%. It is observed that about 60-65% flowers are processed during rainy season (July-Sept), 15% in the winter season (Nov-Jan) and rest during summer season (March-June).[3] The male inflorescences are valued for the fragrance emitted by the tender white spadices covering the flowers and for the valuable attars obtained from them. The commercial exploitation of male spadices is centered mostly in and around Ganjam district of Orissa and some parts of Madras.[4] The kewda oil, attar and water are used in the perfumery and flavour industry. It blends well in all types of perfumes to produce scenting clothes, bouquets, lotions, cosmetics, soaps, hair oils, tobacco and agarbati. The kewda water is used for flavoring various food materials, sweets, syrup and soft drinks.[4] The present survey has been conducted for last 3 years in the context of flower production and its utilization, total area and cost benefit ratio of flower processing and production.

Materials and Methods

The survey was conducted giving detailed questionnaires in all kewda growing areas covering the aspects of

1. Flower production and utilization
2. Kewda canopy area
3. Economic benefit by production and benefit.

Result and Discussion

The detailed survey of Ganjam district showed that wild growth of kewda is concentrated along the coastal belt. Ganjam district lies between 19^0-10^0 to 20^0-21^0N Latitude and 84^0-85^0E Longitude covering an area of about 8,00,000 hectares and about 80 kms of sea coast touching the Bay of Bengal. It spread from Rishikulya River in the north to Sarala village in the South over a stretch of 40 km coastline. It covers 39 Gram panchayats in four blocks of Ganjam districts namely Ganjam, Chatrapur, Rangeilunda and Chikiti (Table-1).

About 80-85% of kewda canopy is concentrated within 10 km radius from the seacoast. It is estimated that about 5,000 hectares of land constituting 6% of total land area in Ganjam district is covered under kewda canopy. In Ganjam district, the block i.e. Chatrapur and Rangeilunda covers 79% of the kewda plantation area and the rest by the Ganjam and Chikiti blocks. In Ganjam district, the area under kewda

plantation four blocks namely Ganjam, Chatrapur, Rangeilunda and Chikiti covers 20%, 56%, 79% and 17% of the total geographical area respectively. Though the total geographical area of Chatrapur is highest, the kewda plantation area is more in Rangeilunda block (Table-1). Although flowering of kewda takes place throughout the year, 70-80% flowers become available in rainy season (June-September); 10-15% in winter (November to January) and rest in summer i.e. during March-May.[5] In Ganjam district, it is estimated that 3,00,000 to 4,00,000 trees produce approximately 1,00,00,000 numbers of flowers per day of which only 10% is utilized for production of essential oil, attar and water. Rests 90% of the flowers remains unutilized and are destroyed in the field. Now there are 117 number of distillery units, each of them having 7-8 number of still/degs/bhatti. The total number of stills or degs is around 950 out of which 51% is present in the Rangeilunda block (Table-2).

There was peculiarity seen in the production of kewda oil and attar. Though the number of flowers utilized for production of attar was maximum in Rangeilunda block. The reason behind is that people of this area go for the production of kewda oil and not attar and this block produces around 40% of the total oil production. The number of distillery units and the still or degs present per distillery unit are more in Rangeilunda block followed by Chatrapur, Ganjam and Chikiti block (Table-2). The price of flower which was Rs. 2.00/piece in 1994 has risen up to Rs. 7.00 in 2005 during this 10 years. The cost of collection and processing of flowers and different inputs such as distillation charges, cost of sandalwood oil and the value of the products obtained are presented in the Table-3. A huge number of flowers

Table-1

Details of area and Gram Panchayats involved in Kewda production

Sl. No.	Items	Name of the blocks of Ganjam District				Total
		Ganjam	Chatrapur	Rangeilunda	Chikiti	
1.	Total geographical area (ha) calculated as per map scale	22,615	23,412	21,149	19,537	86,713
2.	Area of kewda plantation (ha)	4,697	13,328	16,819	3,468	38,312
3.	Kewda canopy (ha)	136	1,849	2,635	312	4,932
4.	Total no. of Gram panchayats	10	17	21	13	61
5.	Gram panchayats covered with kewda plantation	3	12	18	6	39

Table-2

Survey data on Kewda flower utilization and production

Sl. No.	Item	Ganjam	Chatrapur	Rangeilunda	Chikiti	Total
1.	Flowers utilized (in lakhs)	28	156	132	20	336
2.	Production of kewda rooh/oil (kg)	78	154	186	52	470
3.	Production of kewda attar in sandal wood oil and DOP (kg)	276	6,218	1,854	123	8471
4.	No. of Distillery units	14	41	55	7	117
5.	No. of Stills (degs)	83	315	487	65	950

are produced in a limited period of time and only 10% of the total flowers are utilized for the production of oil and attar. The current market value of Kewda oil is Rs. 2.5 lakh/kg, Kewda attar is Rs. 20,000/kg and that of Kewda water is Rs. 300/kg. The rate is increasing with the market demand for the products.

Conclusion

The demand for kewda oil and attar has gone up several folds due to its popularity in pan masala and several brands of scented tobacco manufactured in the country.[6] The market demand of oil and attar has increased the number of production units in Ganjam district. The establishment of more industries in the district shall facilitate the employment generation and consequent economic upliftment of the local people. The main raw materials for the kewda industries are collected from wild sources. A major financial input is towards cost of procurement of flowers and the processing. During the last 5 years some progressive farmers have taken up cultivation and developed agro-techniques for its practices. For the sustainable development of this eco-friendly kewda industry, for socio-economic growth of the local people with employment generation on permanent basis, it is necessary to take up systematic cultivation of kewda in the neighbouring areas. It is also necessary to increase the productivity and quality of materials and processing products using improved/quality planting materials, taking up new agro-technology, improved method of distillation, and research and development in quality control and product characterization. Kewda industry not only influences the economy of the area but also stabilizes the ecosystem and sustainable development of that area by utilizing the waste land for kewda plantation.

Acknowledgement

The authors are thankful to Department of Biotechnology, Govt. of India for providing financial assistance to carry out the work and the Director, Regional Research Laboratory, Bhubaneswar for providing necessary laboratory facilities and permission to publish this paper.

Table-3

Cost benefit ratio of Kewda utilization and production

Sl. No.	Items	Four blocks of Ganjam District				Total
		Ganjam	Chatrapur	Rangeilunda	Chikiti	
1.	Flowers utilized (in lakhs)	28	156	132	20	336
	@ Rs. 7/- per flower	196	1092	924	140	2352
2.	Distillation charges (in lakhs) @ Rs. 300/- per 1000 flowers	8.40	46.80	39.60	6.0	100.8
3.	Sandalwood oil (in kg) cost of absorbent (in lakhs)	216	4,268	1,654	122	6,260
	@ Rs.10,000/- per kg	21.6	426.8	165.4	12.2	626.0
	Total Expenditure (in Lakhs)	226.0	1565.6	1129.0	158.2	3078.8
4.	Kewda oil (in kg) value in lakhs	78	154	186	52	470
	@ Rs. 2.5 lakhs/- per kg	195	385	465	130	1175
5.	Kewda attar with absorbent (in kg)	276	5,726	2,647	122	8,771
	value in lakhs @ Rs. 20,000/- per kg	55.2	1145.2	529.4	24.4	1754.2
6.	Kewda water (in kg) value	-	72,316	28,262	-	100,578
	in lakhs @ Rs. 300/- per kg	-	216.948	84.786	-	301.734
	Total Value (in lakhs)	250.2	1747.148	1079.186	154.4	3230.93

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Growth of Oyster mushrooms from two different environments of Orissa

J.A. Swamy and P.K. Behera
P.G. Department of Botany
Berhampur University, Berhmpur-760 007, Orissa

Growth of *Pleurotus sajor-caju* and *P. florida* collected from two different mushroom cultivation units located at sea shore and in a forest was compared. Growth of *Pleurotus sajor-caju* was almost twice that of *P. florida* under both the environmental conditions. Time to 50% growth of *Pleurotus sajor-caju* was faster under all ranges of temperature at the forest site environment and was slower in *P. florida* at both the sea shore and forest sites. Maximum yield was recorded in *Pleurotus sajor-caju* at the forest site at 20±2°C. Difference between two species of *Pleurotus* were consistent in their growth at separate environmental conditions of the cultivation site.

Key words: *Pleurotus sajor-caju, P. florida*, sea shore, forest, Orissa

Introduction

Environmental conditions are known to regulate the growth of mushrooms. In the world around 22 edible mushrooms are cultivated commercially (Chang and Miles, 1991; Rajarathnam *et al.*, 1992). The environmental conditions of Orissa favours for the cultivation of only two edible mushrooms such as paddy straw (*Volvariella*) and oyster (*Pleurotus*). Two closely related species of *Pleurotus* are grown widely in the winter season in Southern region of Orissa for their higher growth and better yield (Bihary *et al.*, 2002). High fructification of edible mushrooms in forests of Orissa has been reported during rainy season (Swamy *et al.*, 2003). Therefore a study was conducted to determine whether the two species of *Pleurotus* differed in growth when cultivated under the two different environmental conditions.

Materials and Methods

Pleurotus sajor-caju and *Pleurotus florida* were collected from Mushroom cultivation units located in two different environments, e.g. in the sea shore and in a forest and their fresh weights were determined. Ten samples of each species from each location under three different temperature e.g. 20, 25 and 30°C were sampled for 50% growth also the time period was recorded. Data from each harvest and location were subjected to two way analysis of variance with species and temperature regime following Gomez and Gomez (1984).

Results and Discussion

Fresh weight of *Pleurotus sajor-caju* was higher and weight was almost twice as much as those of *P. florida* at both the locations (Table-1). Fresh weight of *P. sajor-caju* from forest site was significantly higher than those from sea shore site. The differences may be attributed to their response to differences in the environmental conditions as the species are different.

The time to 50% growth of both species was quicker in forest site at temperature 20±2°C (Table-2). The time to 50% growth was shorter for *P. sajor-caju* than for *P. florida* under all temperature regimes and was also not location specific. The shortest time to 50% growth of *P. sajor-caju* at 20±2°C to forest site may be related to favourable environmental condition to that of *P. florida*. Similar results are reported earlier by Tripathy (1999-2003), Bihary (2003) and Jyoti Kanchan (2004).

At 18 days after fructification, growth of *P. sajor-caju* was significantly higher and had more weight in forest site than *P. florida* of sea shore site under two temperature regimes (Table-3). At 23 and 28

day fructification stage the production of both species declined steadily under both the temperature regime and the locations. Differences between both the species in fructification and biomass production were significant but was more pronounced at 20±2°C on the 18th day of fructification. These findings are in agreement with the earlier studies (Swamy, 2000; Tripathy, 2003; Swamy *et al.*, 2003 and Jyotikanchan, 2004).

The results showed that time to 50% growth and relative time of fructification and production rate are important factors in determining competitive

Table-1

Fresh weight of *P. sajor-caju* and *P. florida* grown in two different locations in Orissa

Location	Name of the place	*P. sajor-caju*	*P. florida*
		g bed^{-1}	
Sea shore	Gopalpur-on-Sea	610.20 ± 2.6^{a}	340.68 ± 4.5^{c}
Forest	Dakhinapur	746.40 ± 3.8^{b}	370.86 ± 3.2^{c}

Mean ± S.E. based on ten replicates. Values followed by the same letter are not different ($p \geq 0.05$) based on t-tests.

Table-2

Time to 50% growth (in days) of *P. sajor-caju* and *P. florida* under three temperature regimes in the sea shore and forest sites of Orissa

Temperature (°C)	*P. sajor-caju*		*P. florida*	
	Sea shore	Forest	Sea shore	Forest
20 ± 2	10.2 ± 0.6	8.4 ± 0.2	10.6 ± 0.2	9.2 ± 0.5
25 ± 2	12.5 ± 0.4	10.6 ± 0.4	12.8 ± 0.6	10.8 ± 0.3
30 ± 2	14.2 ± 0.3	13.2 ± 0.5	15.0 ± 0.4	13.8 ± 0.4

Mean ± S.E. based on ten replicates.

Table-3

Fresh weight (g) of *P. sajor-caju* and *P. florida* growth under two temperature regimes in the sea shore and forest sites of Orissa

Temperature (°C)	Days after fructification	*P. sajor-caju*		*P. florida*	
		Sea shore	Forest	Sea shore	Forest
20 ± 2	18	605.8 ± 4.2	720.6 ± 2.4	380.6 ± 2.7	405.7 ± 3.8
	23	485.6 ± 2.8	510.8 ± 3.6	192.4 ± 3.6	320.2 ± 5.6
	28	308.4 ± 3.4	350.4 ± 2.2	110.8 ± 4.4	204.6 ± 6.2
25 ± 2	18	590.4 ± 4.6	680.4 ± 3.2	206.7 ± 2.6	390.8 ± 2.4
	23	380.6 ± 3.2	410.6 ± 4.5	200.2 ± 1.8	370.6 ± 3.2
	28	275.4 ± 3.4	305.2 ± 3.8	103.4 ± 1.2	188.4 ± 2.7

Mean ± S.E. based on ten replicates. All species comparisons were significant at ($p \geq 0.05$)

ability of both species which would be beneficial for predicting yield reduction due to the differences in location and temperature regimes.

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In vitro propagation of *Catharanthus Roseus* G. Don.: A potential medicinal plant

Priyambada R. Biswal, A. Swain*, T.K. Dash*, P.K. Swain and S.P. Rath

Cytogenetics and Plant Biotechnology Laboratory, P.G. Dept. of Botany,
Utkal University, Bhubaneswar-751004 (India)
*Department of Botany, S.V.M. Autonomous College, Jagatsinghpur

An effiecient protocol for *in vitro* propagation of *Catharanthus roseus* G. Don. (Apocynaceae) has been established. Axillary buds were cultured on Murashige and Skoog's (1962) basal medium fortified with various combinations of cytokinin and auxin either alone or in combinations. Callus and shoot formation from the axillary buds of *Catharanthus roseus* was initiated with combinations of auxin α-naphthalene acetic acid (NAA) 2.0 mg/l and cytokinin 6-benzylaminopurine (BAP) 0.5 mg/l. This induced an organogenic and chlorophyllus callus with an average of 3-5 shoots per node. Subsequent culture enhanced the multiplication of shoots on MS medium with 0.5-3.0 mg/l BAP. Microshoots were successfully rooted on half strength MS medium with 1.4-2.0 mg/l Indole-3-byutaric acid (IBA) showed thick and stout roots. Eighty percent of the regenerated plantlets were acclimatized and hardened successfully to the natural environment.

Key words: *Medicinal plant, in vitro propagation, Catharanthus roseus, organogenesis, axillary bud.*

Introduction

Catharanthus roseus G. Don., commonly known as periwinkle, belongs to the family Apocynaceae. It is a perennial herb, about 1.0-1.5 metre heights found in sandy coastal areas and commonly available in gardens due to its medicinal values (Fig. A). Screening of medicinal plants in India is going at a very slow rate. However modern science demands meticulous work lying on the drugs at a rational basis to make them more acceptable for the benefit of mankind. Nwafor *et al.* (2001) have screened more than 40,000 plant species for anticancer effects and *Catharanthus roseus* is one of them. The medicinal value of the plant is due to the presence of chemical compounds of Carbon, Hydrogen, Oxygen and Nitrogen in plant tissues like roots, shoots, leaves, flowers, fruits and seeds which produce a definite physiological action on human body (Trivedi and Nehra, 2004). Satapathy *et al.* (2003) reported that the leaf buds of this plant are used for curing diabetes. Kurian (1995) reported the extract of fresh leaf is said to be anticarcinogenic and also used for diabetes and diarrhoea. *Canthranthus roseus* plays as abortive, hypoglycemic, antidiabetic, antidysenteric, purgative, haemostatic and wound healer (Narayana *et al.*, 1977). This plant possesses largest number of alkaloids in the plant kingdom (Hum-Lin and William, 1972). Noble *et al.* (1958) discovered antineoplastic activities of leaf alkaloids. The alkaloid vincristine sufate is employed mainly in childhood leukemia and blood cancer (Conti and Creasy, 1975).

In *Catharanthus roseus*, the propagation through seed is conventional and also there has been an increase in the demand for raw materials. Hence, conservation and rapid multiplication of this drug yielding plant has become imperative. Tissue culture technology can provide an alternative way to substantiate the rapid multiplication of elite clone and germplasm conservation. *In vitro* regeneration of complete plantlets from apical and axillary buds has been reported from number of medicinal species (Bajaj *et al.*, 1988 and Purohit *et al.*, 1994). It was also observed that during the last few years, the application of in vitro culture of endangered medicinal plants through clonal multiplication has been considerably emphasized (Arora & Bhojwani,

1989). Yuan *et al.* (1994) reported shoot regeneration on MS medium supplemented with 7.0 mg/l BA and 1.0mg/l NAA and light intensities of 550-700 Lux were found to be beneficial to the formation of shoots when MS medium was supplemented with 2 mg/l 6-BA and 0-0.1 mg/l NAA.

Attempts have been taken to obtain an efficient system with a short period of time and also a less requirement of plant growth regulators for achieving high frequency of shoot multiplication in *Catharanthus roseus* and to successfully transfer it to natural environment.

Materials and Methods

Nodal explants with axillary buds of *Catharanthus roseus* were collected from the tender parts of mature plants grown in the medicinal plant garden of P.G. Dept. of Botany, Utkal University, Bhubaneswar, India. First they were washed under running tap water, then with a detergent (Teepol, 5% v/v) for 10 minutes and then also kept under running tap water for 30 minutes. The explants were surface sterilized in mercuric chloride solution (0.05%, w/v) for 7 minutes following thorough wash in sterile water. The materials were cut into appropriate sizes (0.5-1.0cms) and cultured on sterile medium.

The basic nutrient medium consisted of MS (Murashige and Skoog, 1962) salts and vitamins with 3% sucrose (SRL), gelled with 0.8% agar (HIMEDIA) with cytokinin (BAP & Kinetin) and auxin (NAA, IBA & 2,4-D) at different concentrations (0.2-2.0 mg/l) either alone or in combinations along with 0.01% myoinositol. The induction of root was done on half strength MS medium with auxins (IBA & NAA) at different concentrations or with combinations. The pH of the medium was adjusted to 5.9 before autoclaving at 15 p.s.i at 125°C for 25 minutes. The cultures were incubated at 25±2°C under 16/8h photoperiod with $35aE^{-2}S^{-1}$ illuminations with cool white fluorescent lights. Rooted plantlets were transferred to plastic pots containing farmyard manure, pure soil and sand (1:1:1) in greenhouse. Ten cultures were raised for each treatment and all the experiments were repeated twice. The percentage of response, mean, SD and SEMs were calculated.

Result and Discussion

Axillary bud multiplication

All the combinations of auxin (NAA, IBA & 2, 4-D) with cytokinin (Kinetin and BAP) produced callus with variable response (Table-1 and Table-2). The strongest response to achieve bud break on MS medium with BAP 0.5 mg/l and NAA 2.0 mg/l. Node explants produced an average of 3-5 shoots (Fig. B). Single node explant induced an average of ten shoots in presence of MS medium with BAP 1.5 mg/l. According to Marks and Sympson (1994), callus formation may be due to the action of accumulated

Table-1

Efficiency of MS medium fortified with different growth regulators on axillary bud multiplication of *Catharanthus roseus*

Growth regulators (mg/l)			Percentage of response	Number of shoots/Node
BAP	Kinetin	GA_3		Mean ± S.E.M
1.0	0.5	0.2	100	4.8 ± 0.3
1.5	0.5	0.2	100	5.2 ± 0.3
1.5	1.0	0.2	90	3.8 ± 0.4
0.5	1.0	-	100	5.0 ± 0.3
1.5	-	-	100	5.8 ± 0.2
3.5	1.5	-	80	5.4 ± 0.2
-	2.0	0.2	70	3.0 ± 0.4

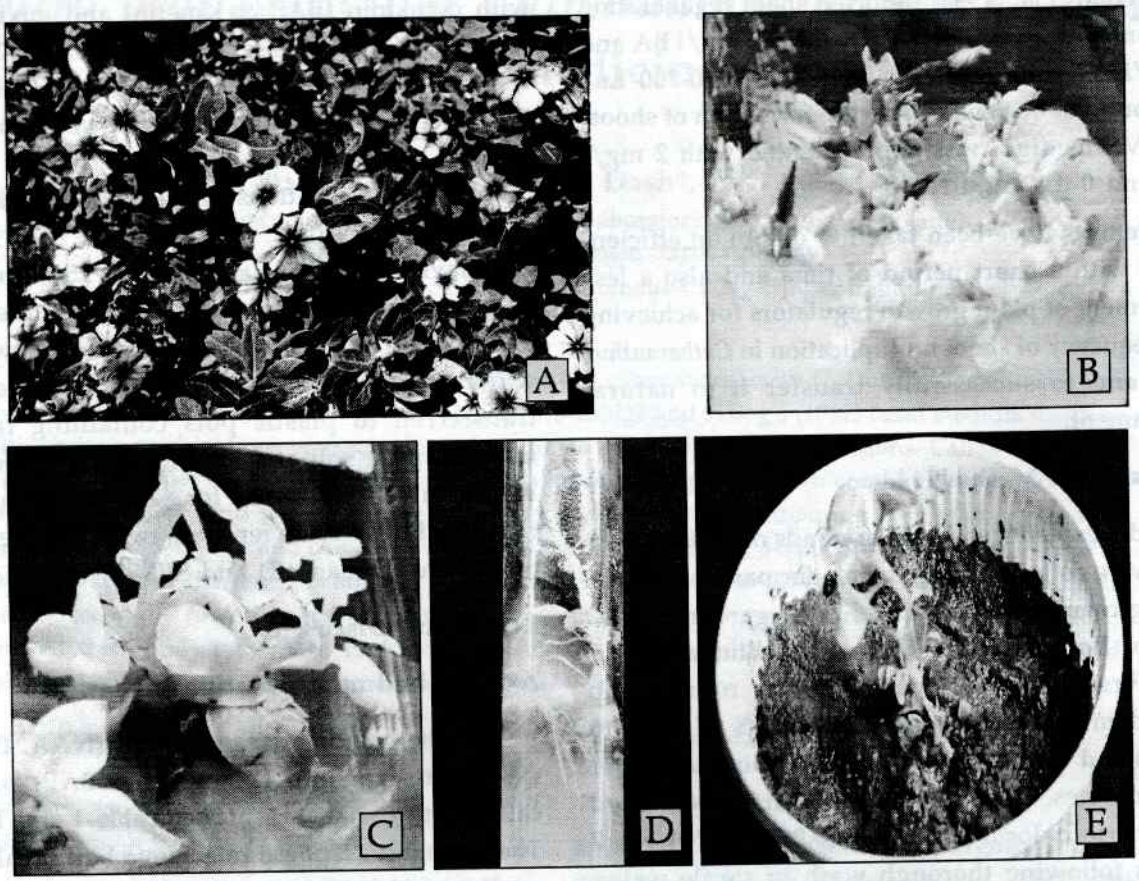

Fig. A : A full grown plant of *Catharanthus roseus*

Fig. B : Shoot regeneration in callusing medium in *Catharanthus roseus.*

Fig. C : Culture showing multiple shoots

Fig. D : Isolated shoot-lets showing rooting

Fig. E : Regenerated plants maintained in green house in potting mixture.

Table-2

Efficiency of ½ strength MS medium with different growth regulators on rooting of *Catharanthus roseus*

Growth regulators (mg/l)		Percentage of Response	Average number of Roots/Shoot Mean ± S.E.M	Length of roots/shoot Mean ± S.E.M	Nature of Roots
NAA	IBA				
-	2.0	100	3.2 ± 0.2	5.6 ± 0.2	Long and sturby
-	1.6	100	4.0 ± 0.3	5.4 ± 0.5	Long and sturby
-	1.4	90	4.8 ± 0.3	5.8 ± 0.3	Long and sturby
-	0.6	90	3.6 ± 0.5	6.2 ± 0.7	Long and sturby
0.5	0.5	80	4.0 ± 0.3	3.7 ± 0.4	Thick short callus at base

auxin at the basal cut end, which stimulates cell proliferation, especially in the presence of cytokinin. Callus developed on MS medium with BAP and NAA was hard, nodular in texture and friable white in colour. The present study exemplifies a positive mode of shoot induction efficacy by low concentration of an auxin in combination with cytokinin. Excision and culture of the segments from the *in vitro* derived shoots facilitated the development of increased number of shoots. The shoot multiplication at an enhanced pace which was achieved by subsequent cultures up to three cycles.

Indirect Organogenesis

Node callus that developed on MS medium with BAP 2.0 mg/l underwent organogenesis. Nodal callus developed an average of 10 shoots (Fig. C, Table-1). Subculturing of organogenic callus developed an average of 20 shoots. This high morphogenic efficacy of node callus may be due to the passage for some internodal components from the pre-existing axillary buds that are essential to evoke caulogenesis (Martin, 2002). After 30days, regenerated shoots exhibited abscission of leaves and shoot tips. However, axillary buds just below the point of abscission of the shoot tip initiated and resumed growth. This is in agreement with results obtained for *Hemidesmus indicus* (Patnaik and Debata, 1996).

***In Vitro* Rooting and Acclimatization**

Well developed shoots developed through axillary buds or shoots derived from callus were excised and cultured on half strength MS medium with different concentrations of auxins for rooting. IBA was found more suitable for root induction than NAA. Shoots obtained from the axillary shoot base callus rooted on MS medium containing 1.6-2.0mg/l IBA. The shoot cultured on half strength MS medium with 2.0mg/l IBA showed best result in 10-14days and each shoot developed an average of 7-8 thick and stout roots (Fig. D, Table-2). The shoot transferred on to MS medium with combination of auxins (NAA & IBA) showed callus at the base and only 1-3 roots were observed.

The effectiveness of IBA in rooting has been reported in many species by different authors. According to Epstein *et al.* (1993), IBA was showed to be taken up better than other auxins in cell suspension culture of *Petunia hybrida*. Ludvig-Muller (2000) observed that transport velocity of IBA was slower compared to that of IAA and NAA. The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus its better function in inducing roots (Nickell, 1982)

Plantlets were transferred directly to small plastic pots filled with sterile farmyard manure, pure soil and sand in the ratio of 1:1:1. Growth revived after 14days of transplantation (Fig. E). Among 70 plantlets transformed to soil, about 80% were found to survive and the plantlets exhibited morphological characters similar to those of the source plant.

The protocol described here for the micropropagation of *Catharanthus roseus* facilitates rapid propagation of this important as well as valuable medicinal plant. Furthermore, this protocol can be used to provide a tool for further research in procuring the important alkaloids; those include vincristine, vinblastine, pseudoindoxyl alkaloid-rosamine, β-carboline, bannucine and leurosinone. These alkaloids have got tremendous phramaceuitical importance, which helps for curing hypertension, diabetes and for leukemia.

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In vitro plant regeneration from nodal explants of *Phyllanthus amarus* Schum. & Thonn. : An important medicinal plant

L.P. Mishra* and S.P. Rath

P.G. Department of Botany, Utkal University, Bhubaneswar, India

*Department of Botany, Raj Sunakhala College, Nayagarh

Phyllanthus amarus (Euphorbiaceae), an important medicinal plant, is used for control of diabetes, hepatitis B and in the treatment of many other health problems. With the aim of rapid micropropagation of *Phyllanthus amarus*, the nodal segments of the matured plants were cultured on MS medium supplemented with different concentrations of BAP (0.5 mg/l to 4.0 mg/l) alone and along with Kinetin (0. 5 mg/l). Among all the treatments MS + BAP 1.0 mg/l has given best result. The aseptic nodal segments cultured on MS medium supplemented with BAP 1.0 mg/l produced 8 shoots per explant which on sub culture was increased to 13 shoots. The individual *in vitro* shoots produced roots when they were cultured in ½ MS medium without any growth regulators.

Key words: BAP, hypoglycaemic, micropropagation, nodal explants, *Phyllanthus amarus*

Introduction

Phyllanthus amarus Schum. & Thonn. (Euphorbiaceae) is an herbaceous medicinal plant widely distributed in the tropics. Species of *Phyllanthus* are used in traditional medicines for the treatment of various ailments. Several reports are there on the hypoglycaemic activity of *P. amarus* (Moshi *et al.*, 1971; Moshi *et al.*, 2001; Raphael *et al.*, 2002; Srividya & Periwal, 1995). As a medicinal plant, importance of *P. amarus* was further increased with reports of its anti tumor and anti-carcinogenic activities and its potential as a remedy for hepatitis B viral infection (Rajeshkumar *et al.*, 2002). *P. amarus* was also found to have anti-oxidant and hepatoprotective properties and anti inflammatory potential (Kiemer *et al.*, 2003). Several chemical compounds were identified from this plant such as amariin, amariinic acid and Phyllanthusiin D (Foo and Wong, 1992; Foo, 1993 and 1995).

The traditional practitioners usually obtained the whole plant for the preparation of traditional medicine through cultivation or collection from the wild. However, cultivation of this plant often encountered with low seed germination and seed heteromorphy (Unander *et al*, 1995). Hence in vitro culture techniques could be used as alternatives for the production of the plant to meet the market demand. However very few works have been done on micropropagation of this plant. In the present work, a suitable protocol has been established for micropropagation of *P. amarus* Schum & Thonn. through multiple shoot induction from nodal explants.

Materials and Methods

Shoots of *P. amarus* (Fig. 1) of about 5-7 cm long with 4-5 nodes were collected from the medicinal plant garden of Botany department, Utkal University, Bhubaneswar, India. The shoots were first rinsed under running tap water for 30 minutes. Then they were washed with 5% teepol and again were rinsed under running tap water for 30 minutes. Then they were surface sterilized with 0.1% $Hgcl_2$ for 5 minutes followed by rinse with sterilized distilled water for three to four times under a laminar flow cabinet. After this the shoots were cut into single nodal segments which were used as explants. Then the nodal explants were inoculated in Murashige and Skoog's (1962) basal medium (MS medium)

supplemented with either BAP (0.5 mg/l - 4.0 mg/l) or Kinetin (0.5 mg/l - 4.0 mg/l) alone and BAP (0.5 mg/l - 3.0 mg/l) + kinetin 0.5 mg/l. The medium was adjusted to pH 5.8 before autoclaved at 121°C for 20 minutes. The cultures were maintained in a culture room at 25 ± 2°C with 16 hr photoperiod at 55% relative humidity. For each treatment 10 replicates were taken and each experiment was repeated thrice. After four weeks of culture the percentage of response, mean number of shoots per explant and standard error of mean (SEM) were calculated and were tabulated. The photographs were taken in Kodak D x 6490 model.

Result and Discussion

The surface sterilized nodal explants when cultured on MS basal medium supplemented with plant growth regulators BAP (0.5-4.0 mg/l) or Kinetin (0.5-4.0 mg/l) alone and BAP (0.5-3.0 mg/l) + Kinetin (0.5 mg/l) multiple shoots were produced in many treatments. The numbers of shoots per explant were found to be maximum in BAP 1.0 mg/l (Table 1). Liang & Keng (2006) also reported MS supplemented with BA 1.0 mg/l as the best medium for induction of multiple shoot from nodal segments of *P. niruri* and it induced highest number of multiple shoots (6.6 shoots per explants). Catapan *et al.* (2000) reported that culture medium supplemented with BA, Kinetin (Kn) and 2ip induced a maximum of 4–5 shoots from each explant of *P. caroliniensis*. During the present investigation it was observed that BAP 1.0 mg/l induced about 8 numbers of shoots from nodal explants of *P. amarus* after 4 weeks (Fig. 2). Rajasubramaniam and Saradhi (1997) had also proved that BA was effective for shoot multiplication of *Phyllanthus fraternus*. Ghanti *et al.* (2004) reported that MS supplemented with BAP 0.5 mg/l induced maximum number of shoots in shoot tip culture of *P. amarus*. But when nodal explants were cultured in MS basal medium supplemented with Kn only no multiple shoot was produced. Liang & Keng (2006) reported that nodal segments of *Phyllanthus niruri* did not produced multiple shoots when they were cultured in MS medium supplemented with Kinetin (0.5 mg/l, 1.0 mg/l) without BA. In the present study it was found that number of shoots decreased with increase in BAP concentration from 1.5 to 3.5 mg/l (Table 1) and there was no respond at 4.0 mg/l BAP. It was also found that when kinetin (0.5 mg/l) was added to the MS supplemented with BAP (0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l, 3.0 mg/l) multiple shoots were produced in all treatments except BAP 3.0 mg/l + Kinetin 0.5 mg/l (Table 1). However the numbers of shoots were maximum 3.9 ± 0.37 in BAP 1.0 mg/l + Kinetin 0.5 mg/l (Table 1). Hence MS supplemented with BAP 1.0 mg/l was found to be the best for induction of multiple shoots from nodal explants of *P. amarus*.

Further sub culturing was carried out to maintain the *P. amarus* cultures and to increase the number of plantlets. The nodal segments were subcultured on the same medium i.e. MS medium supplemented with 1.0 mg/l BAP. A high number of multiple shoots (about 13 shoots per explant) were recorded at the first sub culture cycle when they were cultured on the proliferation medium (fig. 3).

Table 1

Effect of Phytohormones on multiple shoot formation from nodal segments of *P. amarus* (after 4 weeks)

Phytohormones (mg/l)		Percentage of response	Mean number of shoots ± SEM
BAP	Kinetin		
0.5	-	90	4.7 ± 0.31
1.0	-	96	7.7 ± 0.27
1.5	-	86	3.5 ± 0.26
2.0	-	76	2.2 ± 0.25
2.5	-	73	1.4 ± 0.17
3.0	-	53	0.6 ± 0.11
3.5	-	46	0.5 ± 0.1
4.0	-	-	-
0.5	0.5	80	2.6 ± 0.24
1.0	0.5	80	3.9 ± 0.37
1.5	0.5	76	2.9 ± 0.31
2.0	0.5	66	1.9 ± 0.26
2.5	0.5	56	1.1 ± 0.18
3.0	0.5	53	0.5 ± 0.09

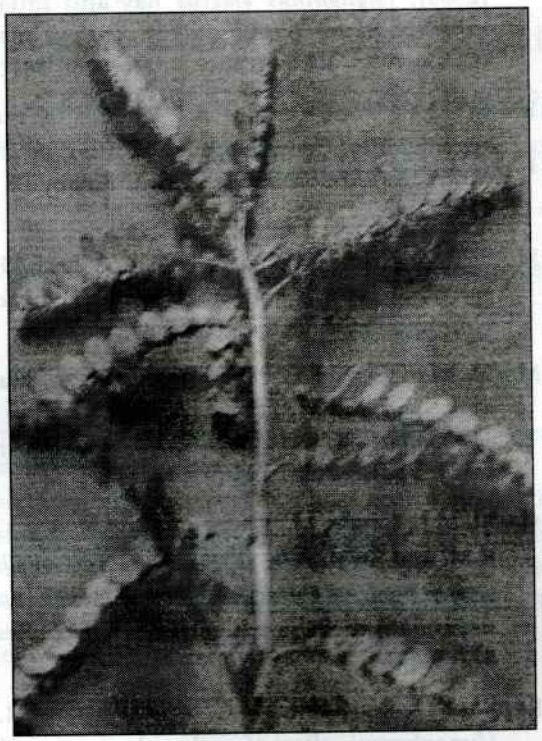

Figure-1

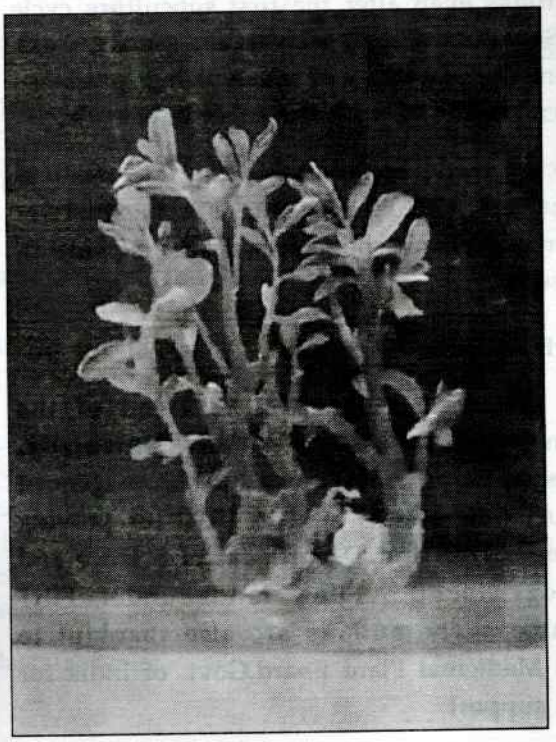

Figure-2

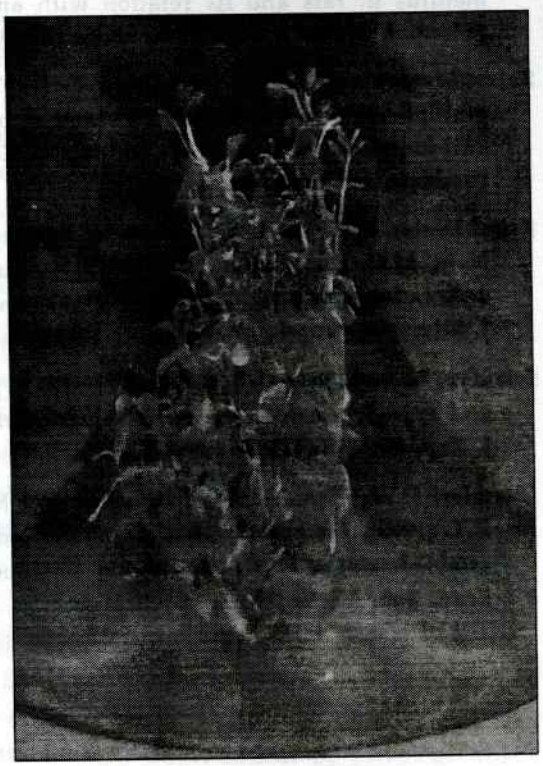

Figure-3

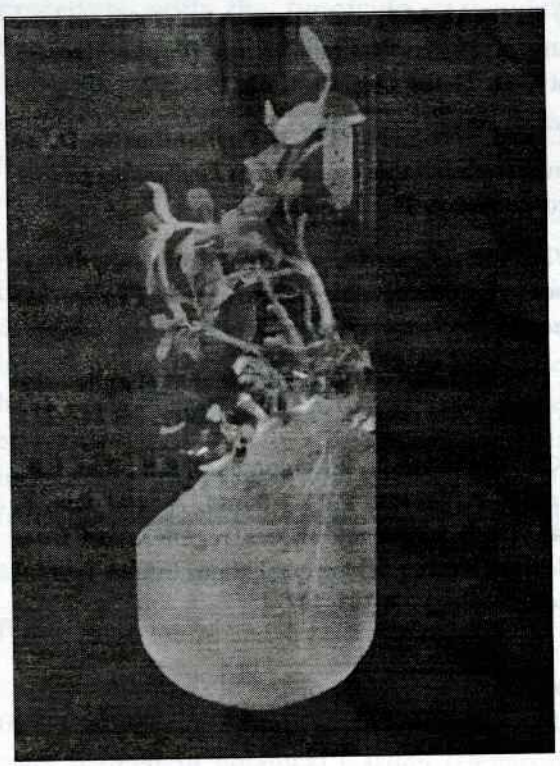

Figure-4

Roots were produced from 50% of the shoot microcuttings even after the first subculture cycle on ½ MS medium (Fig. 4) without any plant growth regulators. The percentage of microcuttings produced roots increased to 85 with the subculture cycle.

Hence it could be concluded that MS medium supplemented with BAP 1.0 mg/l is the best for induction of multiple shoots from nodal explants of *P. amarus*.

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Study of defense-related enzymes by *Fusarium* derived elicitors in Banana against panama disease caused by *Fusarium oxysporum* f. sp *cubense* using different type of treatments

Janki N. Thakker and I.K. Kothari
Department of Biosciences, Sardar Patel University
Vallabh Vidyanagar - 388 120, Gujarat, India

The effect of Fusarium derived elicitors in inducing resistance in banana against panama disease caused by *Fusarium oxysporum* f. sp *cubense* using three different treatments viz. pre-infection, mixed-infection and post-infection was investigated. Changes in the activities of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and phenols in banana leaves after different treatments were measured. Out of three treatments used the pre-infection treatment was found to be the best for induction of defense against panama disease in banana.

Keywords: *Fusarium oxysporum*, Elicitor, Defense related enzymes.

Introduction

Recognition of potential pathogens is central to plants ability to defend themselves against harmfull microbes. To defend themselves against attack from the vast array of viruses, bacteria, fungi, parasitic plants, nematodes, and insects in their environment, plants are equipped with both pre-formed, constitutive chemical and mechanical barriers as well as with inducible defense systems.

Among several fungal diseases in plants, *Fusarium* wilt in banana is a serious and most destructive disease on many cultivars grown in different banana growing regions in the world[1]. *Fusarium oxysporum* f.sp. *cubense*, a highly variable soil borne fungus, is the causative agent of wilt disease[2]. *Fusarium* wilt is a classic vascular wilt disease in which the fungus gets entry to the water conducting xylem vessels, then proliferates within the vessels causing water blockage. Successful infection requires a number of processes such as early plant-host signalling, root surface attachment, degradation of physical host barriers[3]. The typical symptoms include wilting and death of leaves, followed by death of whole plant. There are no effective chemical control measures for the wilt disease and currently practiced corm injection procedure with the fungicide carbendazim is tedious[4]. Several workers have used fungus like *Phytophthora*[5], *Fusarium*[6], *Pythium*[7], *Colletotrichum*[8] as source of elicitors in controlling many diseases in plants.

The aim of the present study was to elucidate the change in activity of defense related enzymes induced in plants due to fungal elicitors with three different types of treatments viz. pre-infection, mixed-infection and post-infection. This investigation will help us in deciding which treatment is the most effective against wilt disease. These could be implemented in practice and plants could be saved from the destruction caused by *Fusarium* wilt.

Materials and methods

Plant Material

Two months old banana plantlets were procured from Cadila Pharmaceuticals (Ahmedabad). Plantlets were planted in Botanical Garden (Department of Biosciences, SPU, Vallabh Vidyanagar). The research plots were supplemented with farmyard manure (2:1). Proper growth of the plants was checked with the

emergence of healthy leaves. These plants were checked regularly.

Maintenance of Fungal Culture

Culture of *Fusarium oxysporum* f.sp. *cubense*, previously isolated in our laboratory[9], was maintained by regular sub-culturing on Potato-Dextrose-Agar (PDA) (Hi media, India) and incubated at 25 ± 2°C at an interval of 10 days.

Elicitor Preparation

Fusarium oxysporum f.sp. *cubense* was maintained on PDA at 25 ± 2°C. For liquid culture of fungus, 8mm agar plug and 3-4 week old culture was inoculated in Potato-Dextrose-Broth. The mycelium was grown at 25°C for 21 days on PDB. Then media with mycelium was autoclaved for 121°C at 15 Ibs pressure for 20 minutes. Autoclaved content was then crushed intensely in the grinder. This was used as elicitor.

Elicitor Treatment

Different treatments of elicitor and fungi were given to the healthy plant material for estimating changes in different biochemical activity.

Three different infection schemes used are as follows :

1. Pre-infection

For this type of infection 12 plants were used. Out of these 12 plants, 6 were selected as control i.e. without any treatments. Rests of the 6 plants were used as experimental i.e. with treatments. In the experimental plants dead fungal material was inoculated (which acts as elicitor) into the roots by exposing them carefully without injuring or damaging them. Similar treatments were given to the control plants but instead of elicitor, water was added. After the treatment, different biochemical assays for defense related enzymes were done regularly. After 9-10 days, both experimental and control plants were treated again. The treatments this time included digging around the roots and adding live Fungus (10^6-10^8 spores/ml) around the roots of both experimental and control plants. Biochemical studies of defense related enzymes were done at an interval of 1 week regularly for 1 month after inoculation.

2. Mixed-infection

For this type of infection 12 healthy plants were used. From these, 6 plants were selected as control and 6 as experimental. Into the roots of experimental plants fungus (10^6-10^8 spores/ml) and elicitor were inoculated simultaneously, by mixing them together in an equal proportion. From the next day different biochemical assays for defense related enzymes were done for 10 days. After this the readings for 1 month at an interval of 1 week were taken for these biochemical studies.

3. Post-infection

For post infection, 12 healthy plants were used; of these 6 plants were selected as control and 6 were selected as experimental. In the experimental plants, the roots were first inoculated with fungus (10^6-10^8 spores/ml). After 24 hours different biochemical assays were carried out for 12 days at an interval of each 24 hours regularly. Later, elicitor was added to both experimental and control plants roots. After inoculation, the biochemical assays were carried out for 1 month at an interval of 1 week regularly.

Biochemical Assay

For the all three different treatments the biochemical assays of polyphenoloxidase, phenols, peroxidase and phenylalanine ammonia lyase were carried out by collecting the samples from Botanical Garden regularly at same interval using Systronics spectrophotometer 106 for spectrophotometric assays. Also for each of the experiments pre-chilled mortar and pestle were used for crushing of leaves into suitable buffers.

1. Estimation of Polyphenoloxidase (PPO)[10]

Fresh leaf tissues were homogenized in phosphate buffer (pH 7.4, 0.1 M) and centrifuged at 15,000 rpm for 10 min. The supernatant was used as enzyme source.

Assay

200 µl of extract was added with 1.5ml of 0.2 M sodium phosphate buffer (pH 7.4) used as blank. Later on 200 µl of 0.01M catechol was added to start the reaction and readings were at 495nm for the time

interval (Δt) required for increase in the absorbance to 0.05.

2. Estimations of Phenols[10]

Fresh leaf tissues were homogenized in 5ml of methanol (80%) and were centrifuged at 15,000 rpm for 15 min. The supernatant was used as enzyme source.

Assay

One ml of methanolic extract was added to 5ml of distilled water and 250 µl of Folin and Ciocalteu's reagent (1N) was added to this and the solution was kept at 25°C. After 3 minutes, 1ml of saturated solution of sodium carbonate and 1ml of distilled water was added and the reaction mixture was incubated at 25°C for 1 hour. For blank similar reagents were added except enzyme. The colour intensity for total soluble phenols was measured at 725 nm. Standard graph was prepared using glucose, and expressed as glucose formed per mg of fresh tissue per minute.

3. Estimation of Peroxidase (POX)[11]

Fresh plant leaves were extracted with phosphate buffer (pH 7.4, 0.2M). The extract was centrifuged at 15,000 rpm for 10 min and supernatant was used as enzyme source. The extract was stored at 4°C until the assay was carried out.

Assay

Three ml of phosphate buffer (pH 7.4, 0.1 M), 50 µl of Guaiacol Solution (20mM Guaiacol in 100ml distilled water) and 0.1ml enzyme extract were mixed. This was used as blank. Then to this 50µl of H_2O_2 solution (12.3mM in distilled water) was added in the reaction mixture and mixed thoroughly. Reading was taken at 436nm at an interval of 10 seconds. Time required (Δt) for the increase in the absorbance was noted until the difference led to 0.05 was noted.

Enzyme activities in units/ml were calculated by the following formula :

$$\text{Enzyme activity units/l} = \frac{3.18 \times 0.1 \times 1000}{6.39 \times 1 \times \Delta t \times 0.1} = \frac{500}{\Delta t}$$

Where, Δt = time interval for enzyme activity.

4. Estimation of Phenylalanine Ammonia Lyase (PAL)[10]

Fresh leaf tissues were homogenized in Borate buffer (pH 5.8, 2.5mM). Poly Venyl Pyrrolidone (PVP) was added equal to that of leaf weight during homogenization. The homogenate was centrifuged at 10,000 rpm for 10 min. Supernatant was used as enzyme source.

Assay

0.5 ml of Borate buffer was taken in a test tube, followed by 0.2 ml enzyme source solution and 1.3 ml of distilled water. Incubation was done at 37°C for 120min. Further reaction was stopped by addition of 0.5ml of 0.01 M HCl. Blank was prepared in which D-phenylalanine was added instead of L-Phenylalanine. Readings were taken at 290nm. Standard graph was prepared using trans-cinnamic acid and enzyme activity was expressed as cinnamic acid from per mg of fresh tissue per min.

Field Observations

After all the three types of the inoculation (pre, mixed and post) the field observations were taken regularly. After 1 month the change in each plant was observed and recorded.

Results

Biochemical Assay

Pre infection

The experimental plants after elicitor inoculation in the field conditions showed no symptoms of the disease and were healthy. After the inoculation of the live fungus, the symptoms were not seen in the experimental plants whereas the symptoms were seen in the control plants.

The phenolic content of banana is normally higher. However, the activity was found highest in the experimental plants (Fig. 1). After one day of inoculation, the concentration of phenol in the experimental plants increased to two fold than the control. On the third day three fold increase in the phenol activity was observed in the experimental plants. While in the control plants it had remained almost constant. After third day the activity in the

experimental plants became constant, but was higher than control. Later on, after the inoculation of live fungus (after seventh day) the level in the experimental plants decreased initially later it remained constant.

The polyphenoloxidase activity in the control plants was more than that of experimental plants. On the fourth day, in the experimental plants activity reached at par with that of control (Fig. 2).

Later on after inoculation of the fungus (after seventh day) into both of the plants, the activity again started to increase in the experimental plants and also in control. After this treatment, the activity reached to its maximum in the experimental plants on 29th day and then became less in quantity. In the control plants the level had also increased after the inoculation of the fungus and reached to its maximum on 22nd day but gradually decreased and ultimately reached a level much lower than that of experimental plants.

Peroxidase activity in both the plants was equal but the concentration in the experimental plants became higher on the 5th and 6th days (Fig. 3). After the inoculation of the fungus (after seventh day), the activity in the experimental plants started to increase and reached to its highest level on 22nd day.

The activity of PAL was found to be highest on 6th day in the experimental plants, while in the control plants the activity remained almost constant till the 7th day (Fig. 4). After the inoculation of the live fungus (after seventh day) the concentration in the control plants increased but was less than that of the experimental plants. In the experimental plants the activity reached a peak on the 36th day.

Throughout the experiment the concentrations of the enzymes were higher than the control.

Mixed infection

In this type of infection, the symptoms were seen in the experimental plants but these were not vigorous. The symptoms were seen after one month of the inoculation. During this infection, the level of the total phenolics was found to be higher in the experimental plants, but an overall constant activity was seen with a gradual increase in the enzyme levels (Fig. 5).

Polyphenoloxidase activity had increased in the experimental plants that reached to its highest activity on 2nd day and after that it became less than the control plants by the 6th day (Fig. 6). On further observation on 7th, 14th, 45th day the experimental plants showed an increase in the activity than that of control plants.

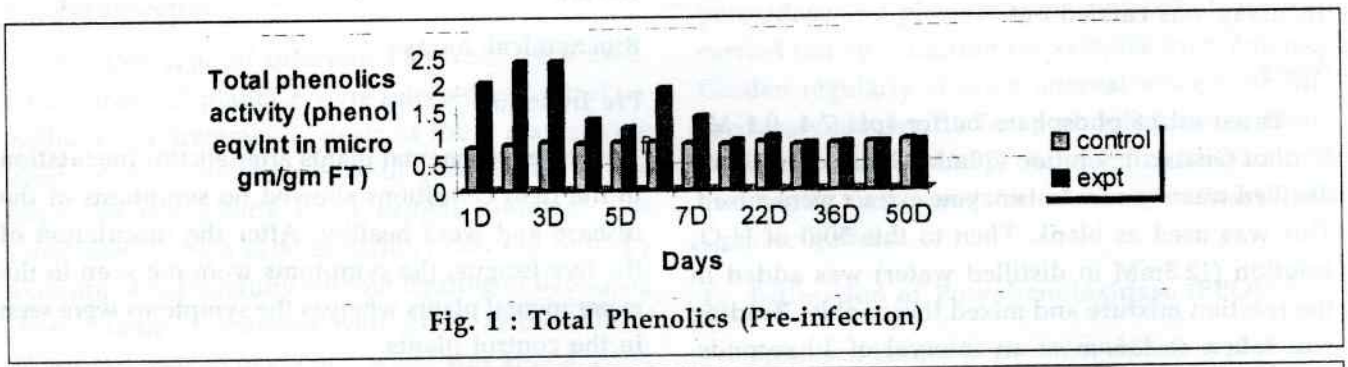

Fig. 1 : Total Phenolics (Pre-infection)

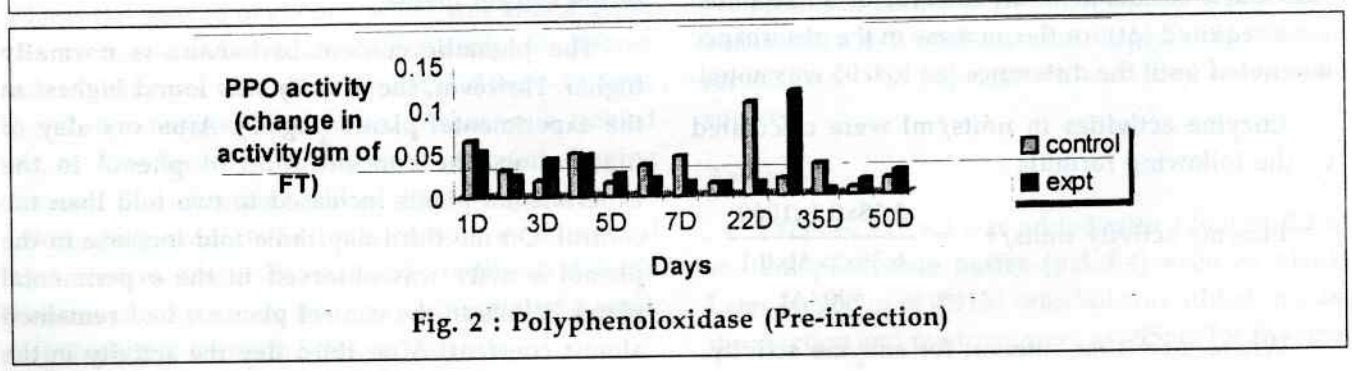

Fig. 2 : Polyphenoloxidase (Pre-infection)

In experimental plants showed more concentration of the peroxidase content than that of the control plants and reached to its highest activity on 3rd day (Fig. 7). After seven days interval the condition remained same i.e. the activity in the control plants was higher than that of the experimental plants. After 7 day a drastic increase observed in the experimental plants till day 31st during which the activity decreases while an overall constant activity was noted for the control plants.

The activity of PAL was found to be highest on 9th day in the experimental plant and in the control plants it was found to be constant (Fig. 8). Later, on the activity decreased in the experimental plants till the 24th day. A sharp increase was observed by 31st day but the activity again receded post 31st day.

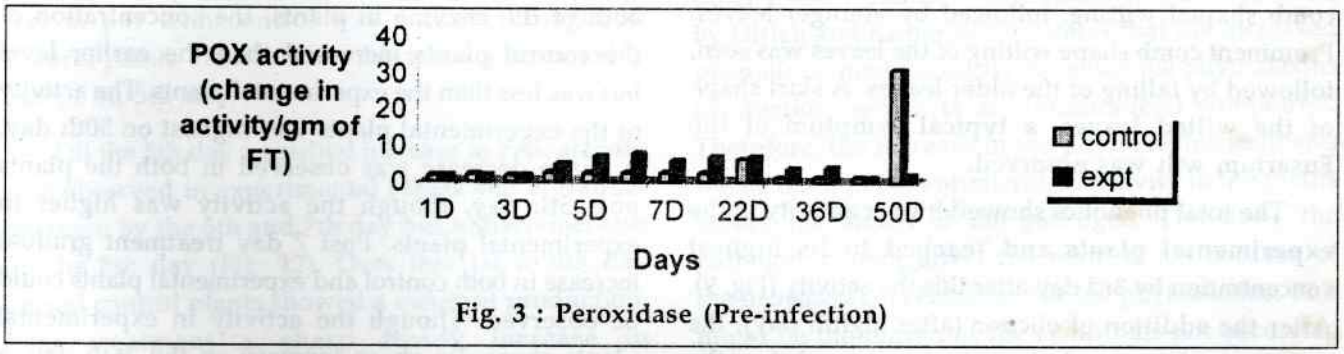

Fig. 3 : Peroxidase (Pre-infection)

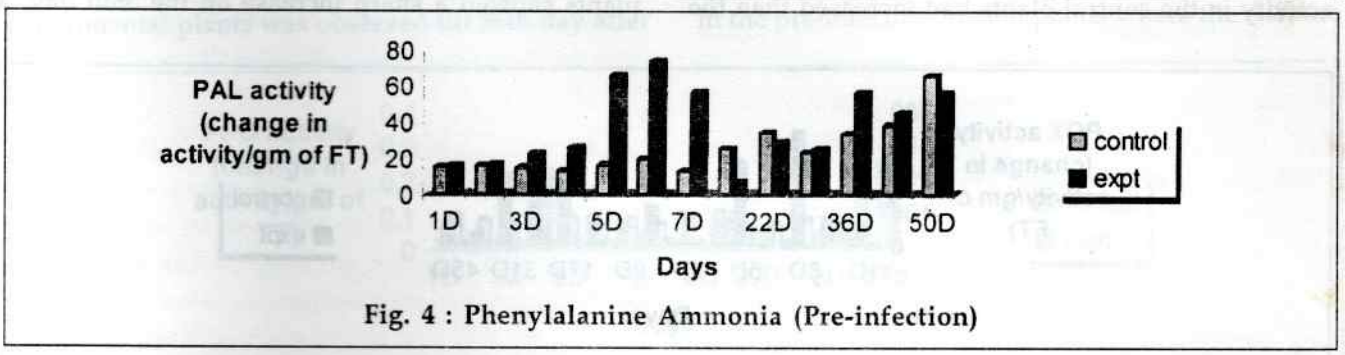

Fig. 4 : Phenylalanine Ammonia (Pre-infection)

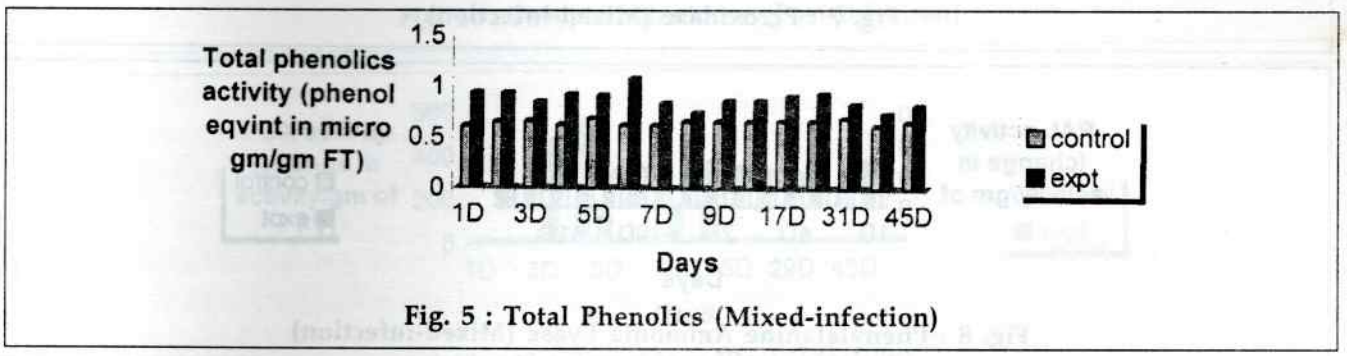

Fig. 5 : Total Phenolics (Mixed-infection)

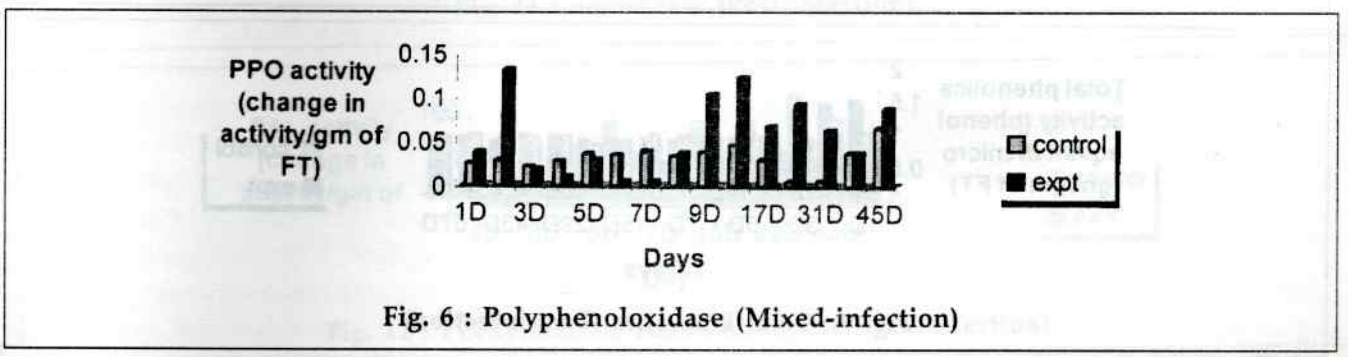

Fig. 6 : Polyphenoloxidase (Mixed-infection)

Post infection

The experimental plants showed characteristic symptoms of the panama disease i.e. discoloration of the leaf margins with yellowish brown spots. Also the youngest leaf showed marginal browning, followed by next leaf showing deepening of the brownish yellow line, proceeding towards midrib in third leaf and finally oldest leaf at bottom showed comb shaped wilting, followed by younger leaves. Prominent comb shape wilting of the leaves was seen, followed by falling of the older leaves. A skirt shape of the wilted leaves, a typical symptom of the Fusarium wilt was observed.

The total phenolics showed higher activity in the experimental plants and reached to its highest concentration by 3rd day after this the activity (Fig. 9). After the addition of elicitor (after eighth day), the activity in the control plants had increased than the earlier concentration however, later on it was almost equal to the experimental plants.

The concentration of Polyphenoloxidase in the experimental was found to be almost equal to the control plants but, the activity reached to its highest on 4th day (Fig. 10). Later on, it again became equal to the control plants and increased little on 7th day. After the addition of elicitor (after eighth day) to both of the enzyme in plants, the concentration of the control plants increased than the earlier level but was less than the experimental plants. The activity of the experimental plants was highest on 50th day. A sharp decrease was observed in both the plants post 5th day, though the activity was higher in experimental plants. Post 7 day treatment gradual increase in both control and experimental plants could be observed. Though the activity in experimental plants showed a sharp increase on the 36th day, a

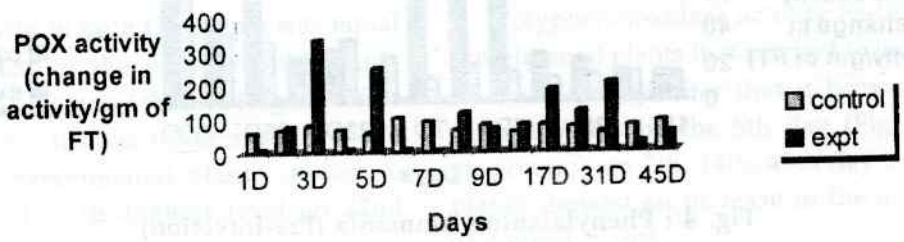

Fig. 7 : Peroxidase (Mixed-infection)

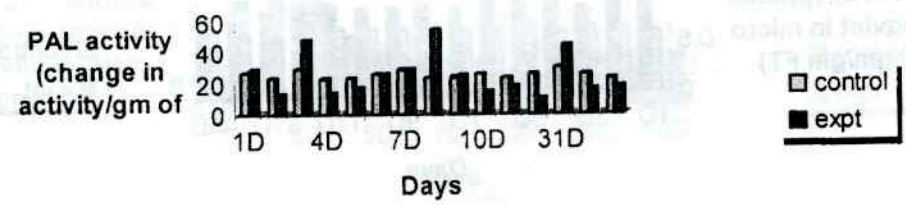

Fig. 8 : Phenylalanine Ammonia Lyase (Mixed-infection)

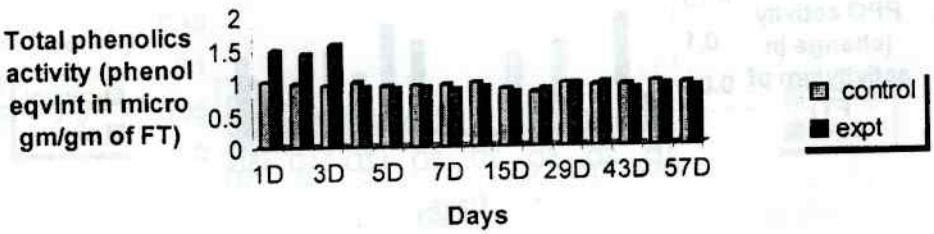

Fig. 9 : Total Phenolics (Post-infection)

huge fall on the 43rd day and a steady sharp increase by the 27th day could be seen.

The activity of the peroxidase was found to increase on 4th day in experimental plant, later on it decreased (Fig. 11). Prior the treatment on the 8th day a sharp increase in the enzyme activity was noticed in experimental plants. But when enzyme activity was assessed thereafter a huge fall which was at par with control was noticed throughout. Control plants did not show this activity remarkably except on 5th day.

Till the 5th day a gradual increase in PAL activity was observed in experimental plants and a gradual decrease by the 6th and 7th day but a sharp increase on the 8th day (Fig. 12). Over this 1st to 8th day period control plants showed a constant production. Post treatment a sharp steady increase in experimental plants was observed till 36th day after which a gradual fall was observed. Post treated control plants had a greater activity than that of experimental plants and reached a peak by 36th day and gradual fall by the 43rd day.

Discussion

The level of the total phenolics content in the pre treated plants was found to be higher than the other treated plants.[12] The result of the work done by Ulrich and Richard[13] indicates that the increased phenolic content indirectly increases the phytoalexins production, which is an antimicrobial compound. Therefore, the increase in the phytoalexins indicates the increase in the antimicrobial activity in the plants before the attack of the pathogen. Therefore, the pathogen cannot affect the plant much because of the increased concentrations of the phytoalexins. This might be the reason of the symptoms not occurring in the pre-infection treated plants where the elicitors

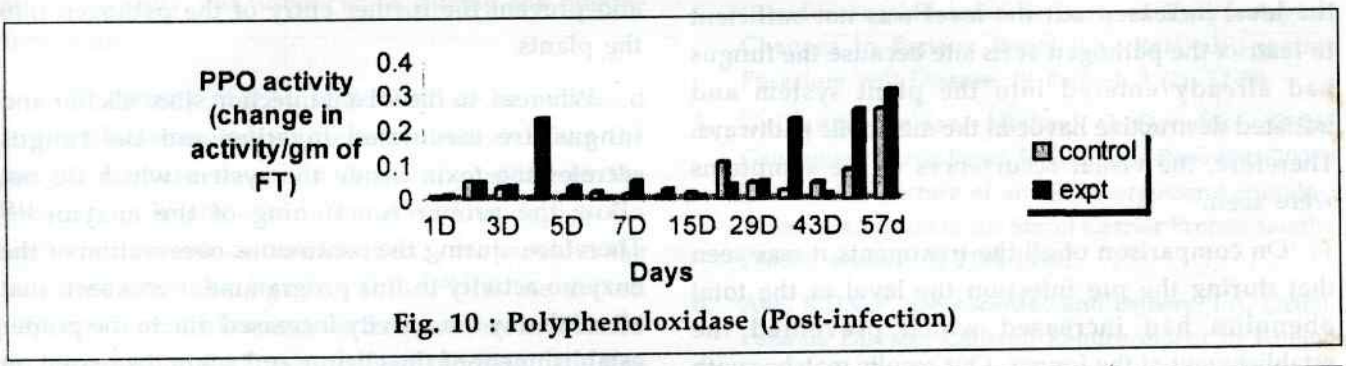

Fig. 10 : Polyphenoloxidase (Post-infection)

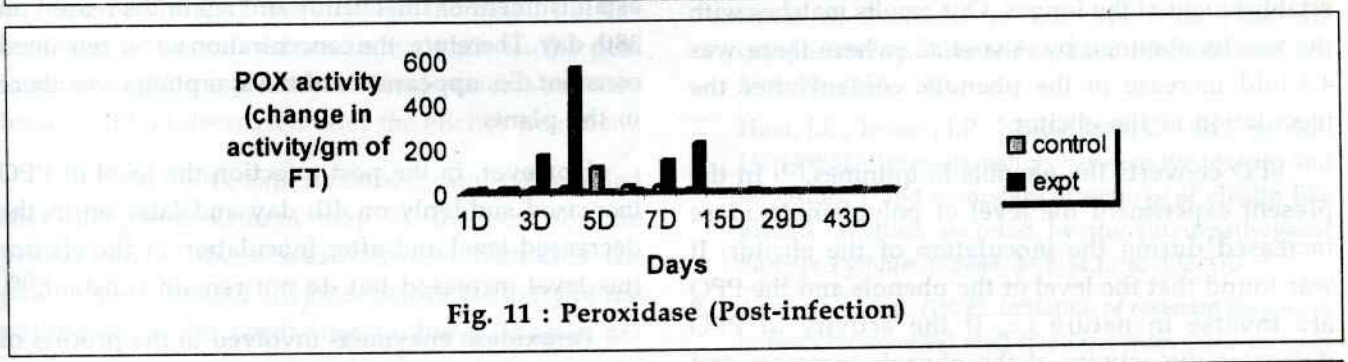

Fig. 11 : Peroxidase (Post-infection)

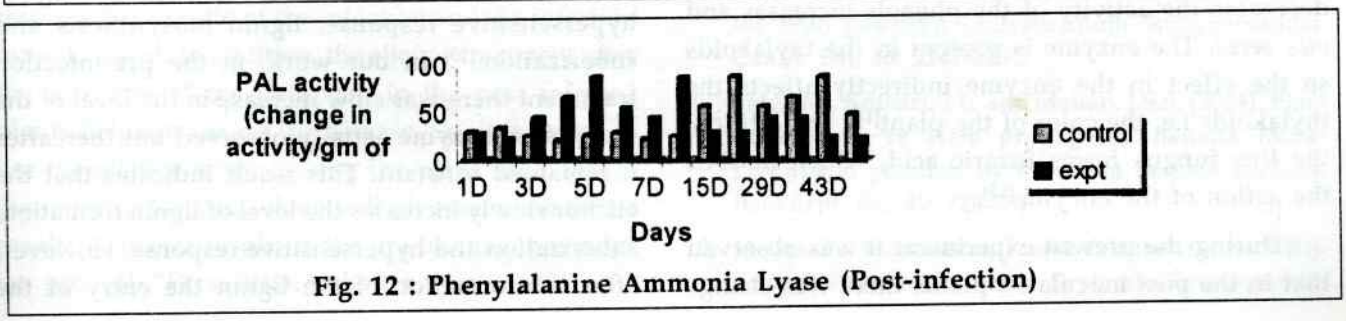

Fig. 12 : Phenylalanine Ammonia Lyase (Post-infection)

were added before the inoculation of the live fungus in the present investigation. In addition, the phenol helps in the formation of the callose and estrification process, which are the plants second line of defense. Therefore, it triggers the plants second line of defense before the entry of the pathogen.[13] Our results corroborate these findings.

In the mixed infection both of them i.e. elicitor and the fungus were inoculated together. There was not much increase in the phenolics level. The present investigation thus suggests that pathogen entry inside the plant was successful and visual symptoms were seen after a prolonged period.

In the third infection strategy, i.e. post infection the fungus had entered into the plant system and had started to spread its infection thread. The phenol concentration had increased at initially but later on it came down because of the successful establishment of the pathogen. After the inoculation of the elicitor, the level increased but the level was not sufficient to restrict the pathogen at its site because the fungus had already entered into the plant system and initiated destructive havoc in the metabolic pathways. Therefore, the visual occurrences of the symptoms were seen.

On comparison of all the treatments it was seen that during the pre infection the level of the total phenolics had increased which prevented the establishment of the fungus. Our results matches with the results obtained by Ana *et al.*, where there was 4.5-fold increase in the phenolic content after the inoculation of the elicitor.

PPO converts the phenols to quinines.[14] In the present experiment the level of polyphenoloxidase increased during the inoculation of the elicitor. It was found that the level of the phenols and the PPO are inverse in nature i.e. if the activity of PPO decreases the activity of the phenols increases and *vice versa*. The enzyme is present in the thylakoids so the effect in the enzyme indirectly affects the thylakoids i.e. the color of the plant[14]. In addition, the live fungus forms fusaric acid, which inhibits the action of the enzyme[15].

During the present experiment it was observed that in the post inoculated plants there was change in leaf coloration. This was because in the post infection the concentration increased suddenly but drastically decreased next day; the imbalance in the enzyme activity affects the functioning of the thylakoids.

So, in the post infection the level of PPO increased but did not remain constant whereas this condition did not arise in the pre infection because instead of the live pathogen, dead component of the pathogen was inoculated. Therefore, in the pre infection the PPO is available for the oxidation of the phenols to convert it into quinones, which is more antifungal in nature than the phenol itself. In addition to this, the enzyme helps in the formation of the superoxide, which is a type of reactive oxygen species acting as a major component during the stressful condition. The superoxide helps the plants to overcome the stressful conditions[16]. This indicates that during the pre infection these compounds were already formed and prevent the further entry of the pathogen into the plants.

Whereas, in the mixed infection since elicitor and fungus are inoculated together and the fungus secretes the toxin inside the system which do not allow the proper functioning of the enzyme.[15] Therefore, during the continuous observation of the enzyme activity in this programme it was seen that after 9th day the activity increased due to the proper establishment of the elicitor and again decreased on 38th day. Therefore, the concentration never remained constant. So, appearance of the symptoms was there in the plants.

However, in the post infection the level of PPO increased suddenly on 4th day and later on in the decreased level and after inoculation of the elicitor this level increased but do not remain constant.[10]

Peroxidase enzyme is involved in the process of hypersensitive response, lignin biosynthesis and suberization.[15] In our work, in the pre-infection treatment there was slow increase in the level of the peroxidase enzyme activity observed but thereafter it remained constant. This result indicates that the elicitor slowly increases the level of lignin formation, suberization and hypersensitive response. However, after the formation of the lignin the entry of the

pathogen is restricted so the entry of the pathogen is not possible. Therefore, in this type of infection there is already presence of the lignin acting as the barrier to the pathogen. In case of the mixed infection treatment the peroxidase level did not remain constant i.e. it increased on 3rd day and later it decreased. After seven days of observation it was again found to increase once and later on, it decreased. In case of the post infection treatment the level increases drastically because the enzyme is located in the tissues of epidermis, sub epidermis and endodermis with lesser amounts in the cortex and stele, so is very sensitive and the entry of the live pathogen was sensed by the enzyme more than that of any other enzymes.[14] Therefore, the level increased suddenly on 4th day and later on, it remained decreased level even after the inoculation of the elicitor. The reason behind this is that the fungus can secrete some lignin-degrading enzymes so the activity did not increase during the elicitor treatment.

The activity of PAL was found to be increased in the plants treated with elicitor first and then the fungus because the plant is more sensitive in the presence of hypoxia and fungus.[17] This depicts the lignifications process since this enzyme provides the precursor for the lignifications.[18] In the mixed infection both elicitor and the fungus were inoculated together so the PAL activity had increased but the level did not remained constant. While in the post inoculated plants the enzyme level was not constant because it had decreased after the elicitor treatment.

From the above experiments, it can be concluded that the pre treatment may be the best for the prevention of the disease since it increases the pathogenesis related enzymes before the entry of the pathogen so the spreading of the pathogen get restricted at the site of the infection and the infection cannot spread. In addition, the elicitor treatment after the infection of the pathogen in the post infected plants helps in curing the disease. So, it is better to use the elicitor while sowing the plant itself before the entry of fungus, which will give us much better results. Our best results in the pre treatment justifies the proverb "Prevention is better then cure".

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Cultural growth on different media and molecular studies of *A. flavus* isolates collected from stored rice of Cuttack district

S. Nayak, S. Mishra and U. Dhua

Plant Pathology Division, Central Rice Research Institute, Cuttack-753006, Orissa

Aflatoxin is a deadly Mycotoxin mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. It is a ubiquitous fungus and mainly grows in regions of high humidity and temperature. Popular Rice varieties were collected from Cuttack district, the climate of which favors mold growth. *A. flavus* were isolated, screened for aflatoxin production and cultural characterization was done. Czapekdox Agar was found to be the ideal media for *A. flavus*. The isolates included in this study preferred sucrose as carbon source. Yeast extract and micronutrient enhanced the fungal growth. The variations in the nutritional requirement of different isolates were also observed. However the genetic variability evident in growth pattern of various isolates on different media was not reflected in the Internal Transcribed Spacer (ITS) region of ribosomal DNA, double digested with two restriction enzymes and banding patterns visualized on agarose gel with Ethidium Bromide staining. Hence finer techniques like Poly Acrylamide Gel Electrophoresis or AFLP to be followed to explore the molecular variability among isolates.

Key words: *Aspergillus flavus, Vegetative growth, Growth Media, ITS*

Introduction

Aflatoxins are potent toxic, carcinogenic, mutagenic, immunosuppressive agents, produced as secondary metabolites by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* on variety of food products and crops such as such as maize, corn, vegetables, spices, chilies, sorghum, groundnut and other oil seeds and rice. Among 18 different types of Aflatoxins identified, major members are aflatoxin B1, B2, G1 and G2. Aflatoxin B1 (AFB1) is normally predominant in amount in cultures as well as in food products. Dietary, respiratory, dermal and other exposures to toxic fungal metabolites produce the diseases collectively called mycotoxicoses while frank growth of fungi on animal hosts produces the diseases collectively called mycoses (Bennett and Klich, 2003). Ingestion of high amounts of aflatoxin may induce lethal effects in poultry animals fed with grain contaminated with the toxin. *Aspergillus* belongs to the Family *Trichocomaceae*, Order *Eurotiales*, Phylum *Ascomycota* and Kingdom Fungi. Colonies of *A. flavus* are green-yellow to yellow-green or green on Czapek's agar. This group of fungi is "degenerate", having lost the ability to form sexual spores. Sclerotia are occasionally produced. A relationship between Mycotoxin production and sporulation has been documented in several mycotoxigenic genera. For example, in *Aspergillus parasiticus* certain chemicals that inhibit sporulation have also been shown to inhibit the production of Aflatoxin. Although they occur more frequently in areas with a hot and humid climate, favorable for the growth of moulds, they can also be found in temperate zones.

Stored paddy and milled rice normally harbor a specific storage myco-flora, consisting of 6-8 osmophilic species of *Aspergillus* and 2-3 of *Penicillium*. Epidemiological studies suggest that the inoculum of these fungi is acquired from the rice field and processing area environments prior to storage. Under normal small-scale storage, fungal numbers gradually fall with time. In large-scale storage, where the grain may become exposed to

unfavorable environments (particularly an increase in moisture content), the fungi grow and cause spoilage (Kalyanasundaram, 1997).

Cuttack is a coastal district of Orissa and a major Rice growing region where so many land races and high yielding varieties are cultivated. Rice seed samples which are used by farmers for consumption were collected from villages of Cuttack district.

Aspergillus flavus have shown varying growth and production of secondary metabolites on different culture media (Criseo *et al.*, 2001). In the present study we have isolated *A. flavus* associated with the above rice samples, screened for aflatoxin producers in a cultural assay method and their growth was studied on three fungal culture media. The rapid growing fungi that overgrow the culture plates and are associated with the samples collected from storage, render counting and isolation of important fungi difficult or impossible. The incorporation of the fungicide Dichloran in the media inhibits such growth (Pitt *et al.*, 1991). Besides observing their nutritional requirements an attempt was made to study the genetic variability, if any, existing among these isolates. The generated information may be useful for developing effective management practices for preventing the aflatoxin contamination in rice and rice based agricultural commodities.

Materials and methods

Rice samples and A. flavus isolates

Different rice varieties such as Khandagiri (Local), Durga (CR123-23), Gayatri (1018), Moti (CR260-136-321), 5656 (Local) and 107 (Local) which were stored in different types of storages and used by farmers for consumption were collected from Nanpur, Bodara and Kalarabanka villages of Cuttack district. However the exact name of some of the varieties couldn't be established as the varieties has been taken in mass scale from the super cyclone affected areas of Cuttack and all the varieties studied in a mass. *Aspergillus flavus* and other associated mycoflora were isolated by blotter plate method (Agarwal and Sinclair, 1996). Isolates were screened for aflatoxin production by a cultural assay method (Saito and Machida, 1999) on β-Cyclo Dextrin Potato Dextrose Agar media (β-CD-6gm (6%), Peeled potato-250gm, Dextrose-20gm, Streptomycin-0.03gm, Agar-15gm and pH - 5.6.). For our study we have taken 6 aflatoxin positive isolates, the passport data is given in Table-1.

Study of vegetative growth pattern

Vegetative growth of isolates was studied on three different media such as: Yeast extract Glucose Trace element media (YGT-1L: Yeast Extract-20g, Glucose-20g, Potato-250g, Streptomycin-0.03g,

Table-1

Passport data of aflatoxin producing *Aspergillus flavus* isolates

L-Low, I-Intermediate, PD-Parboiled Duhusked, RH-Raw Husked

Isolate No.	Rice variety from which isolated	Type of seed	Place	Aflatoxin Production	Date of Collection
AF-5	KHANDAGIRI (Local)	PD	KALRABANK, CUTTACK	L	21-03-05
AF-8	DURGA (CR123-23)	RH	BODARA, CUTTACK	L	21-03-05
AF-9	GAYATRI(1018)	PD	BODARA, CUTTACK	L	21-03-05
AF-11	MOTI (CR260-136-321)	PD	KALRABANK,CUTTACK	L	21-03-05
AF-13	5656 (Local)	RH	NANPUR, CUTTACK	L	21-03-05
AF-20	107 (Local)	RH	NANPUR, CUTTACK	I	21-03-05

Dichloran-2mg, Agar-15g, Trace Element Solution-1ml and pH-5.6), Czapekdox Agar (**CZA**-1L: Sucrose-30g, Sodium Nitrate-2g, Di-Potassium Hydrogen Ortho Phosphate-1g, Magnesium Sulphate-0.5g, Potassium Chloride-0.5g, Ferrous Sulphate-0.01g, Dichloran-2mg, Agar-15g and pH-5.6) and Rice Extract Agar (**REA**-1L: Boiled Rice Extract from 250g healthy rice, Dextrose-20g, Streptomycin-0.03g, Chloramphenicol-0.1g, Dichloran-2mg, Agar-15g and pH-5.6). The periodic growth was observed in terms of colony diameter. Analysis of variance (ANOVA) in growth pattern and Lowest Significance Difference (LSD) determined by CROPSTAT Version 6.1 software.

DNA isolation and PCR amplification of ITS region

DNA from fungal mycelium was isolated by a modified method (Liu *et al.*, 2000). In brief, mycelia of isolates grown in Potato Dextrose broth was blot dried and soaked in lysis buffer (Tris HCl (pH 8.0) 400 mM, EDTA (pH8.0) 60mM, NaCl-150mM, SDS-1%) for 15 minutes and then ground briefly to a fine solution. 150ml of Potassium Acetate was added and mixed thoroughly and centrifuged at >10000rpm for 1 minute. To the supernatant 500µl of Chloroform: isoamyl alcohol (24:1) was added and mixed well by inverting several times. The aqueous phase was separated and double amount of isopropanol was added. The precipitate was dissolved and then centrifuged at >10000rpm for 2 minutes and the pellet was again washed with 70% ethanol. The pellet was air dried and redissolved with 50µl of TE buffer.

PCR of ITS region was done with minor modification (Henry *et al.*, 2000) with ITS-1 and ITS-4 primers (White *et al.*, 1990). The 50µl reaction volume contained 1X PCR buffer, 1.5mM $MgCl_2$, 10 Pico moles from each primer, 0.1mM dNTP and 1U of Taq DNA polymerase. The reaction was carried out in a Thermal cycler (PTC-100, M.J. Research. INC). Forty cycles of amplification were performed after initial denaturation of DNA at 95°C for 4.5 min. Each cycle consisted of a denaturation step at 95°C for 30 s, an annealing step at 50°C for 30 s, and an extension step at 72°C for 1 min, with a final extension at 72°C for 3 min following the last cycle. After amplification, the products were stored at 4°C until used.

Restriction digestion of PCR amplified product

Restriction digestion of the PCR amplified product was done by using two restriction enzymes such as: *EcoRI*, and *MspI* (Mathiyazhagan *et al.*, 2006). The cocktail mixture was prepared as follows: Nanopure water 2.9µl, Buffer Tango (1X) 1.5 µl, Restriction Enzyme 0.3 and amplified DNA 10µl. The restricted products were run on 3% agarose gel for separation of bands and visualized by Ethidium Bromide staining and documented by Gel Documentation System (Vilber Lourmat, France) equipped with Bio-Capt soft ware (Vilber Lourmat, France).

Results and Discussion

The six isolates were obtained from six popular rice varieties (Table-1) and of two different seed types such as Raw husked (AF-8, AF-13 and AF-20) and Parboiled dehusked (AF-5, AF-9 and AF-11). All of the isolates were low aflatoxin producers except AF-20 which produces slight higher amount. This qualitative test is based on the change of yellow colour pigments produced by isolates in culture media into pink when Ammonium Hydroxide is added. Addition of an excess of any acid converts the color back to yellow indicating that the pigments which form the basis for this test act as pH indicator dye. All of these pigments are Anthraquinone intermediates in the aflatoxin biosynthetic pathway (Sinz and Shier, 1991; Bhatnagar *et al.*, 2003).

On three fungal culture media, the isolates showed significant variations in the growth patterns. All the isolates had maximum growth and formed green colour colonies on CZA (Figure-1). Yellow colored colonies were formed on YGT media and whitish brown colonies developed on REA media with the slowest vegetative growth among three media.

YGT and REA both medium had Glucose/ Dextrose but CZA had Sucrose. Potato starch was added to YGT whereas boiled rice extract i.e. Rice starch was present in REA medium. The *A. flavus* isolates included in this study preferred sugar than the starch. CZA had NaNO3, $KHPO_4$ $MgSO_4$, KCl, and $FeSO_4$ besides the carbon source. YGT had trace

elements including Cu and the Copper compounds possess fungicidal properties. However YGT had yeast extract which has vitamins of B complex group and this media was found to be better than the REA. These findings indicate that rice starch is not ideal Carbon source for the growth of *A. flavus*. It was evident from the maximum colony diameter of all the isolates on CZA (Table-2 and Figure-1) followed by on YGT that the micro-nutrients e.g. P, Mg, K, Na, Cl, and S in traces promote the growth of *A. flavus*.

The samples from local market were very dirty and chaff, straw, soils etc. were found to be present in them. Those samples weren't required to be cleaned before analysis in view of the fact that the exact condition for the study existed in them however surface sterilization was done for every sample before blotter plating. Straw contains Cellulose, hemi-cellulose, Ash, silica, Ca, P, Mg, K, Na, Cl, and S (Antongiovanni and Sargentini, 1991). Under congenial conditions the *A. flavus* grows well on these pollutants which infect the grains etc. and obviously

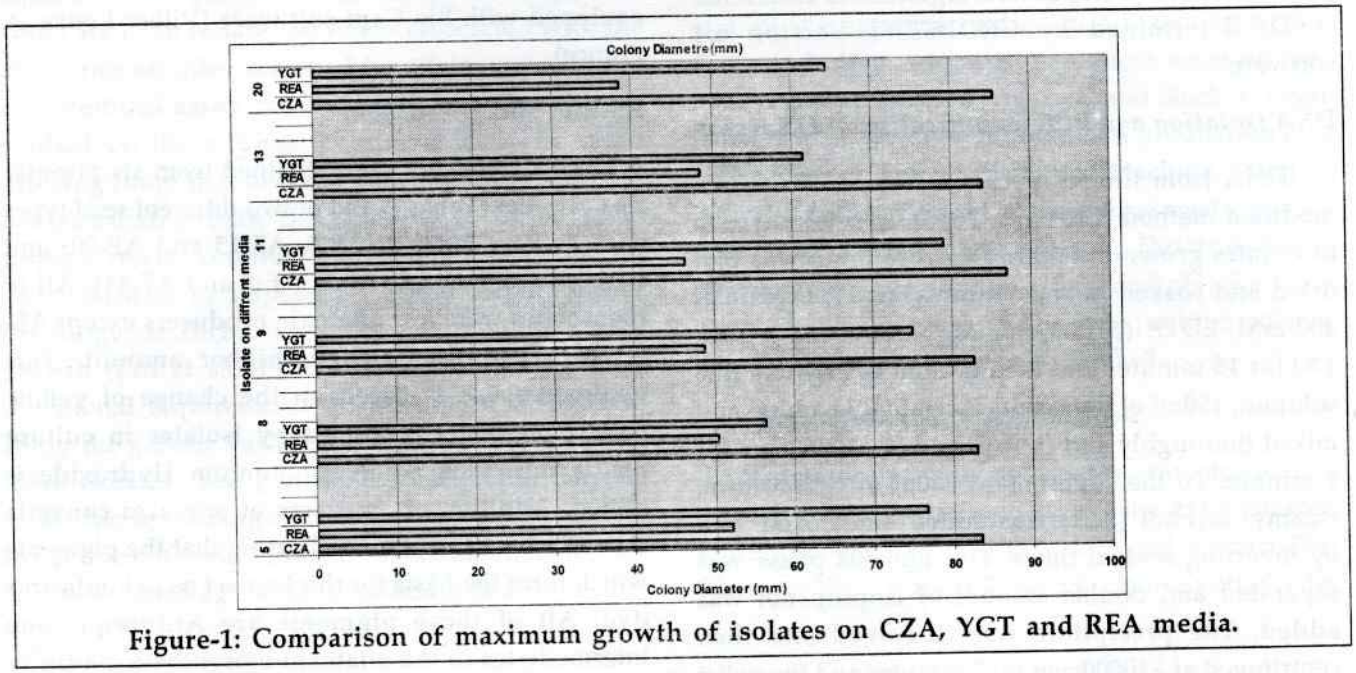

Figure-1: Comparison of maximum growth of isolates on CZA, YGT and REA media.

Table-2

Periodic vegetative growth of isolates on Czapecdox Agar (CZA) medium

Isolate No.	Colony diameter in Days of incubation										Mean diameter/ isolate
	1	2	3	4	5	6	7	8	9	10	
AF-5	0	20.5	39	54.5	68.75	78	83.5	89	91	92	61.623
AF-8	0	9.5	23	35.5	63	76.5	83	89	91	92	56.3
AF-9	0	10.75	32.75	39.25	60.5	74.5	82.75	85.5	89.5	91.5	56.7
AF-11	0	11.5	30.75	38.75	64.5	79	87	91.5	92.5	93	58.85
AF-13	0	9.75	30	39.5	70	80	84	87	91	92	58.32
AF-20	0	21	35.5	50.5	59.5	74	85.5	88	91	92	59.7
Mean diameter/day	0	13.83	31.83	43	64.37	77	84.3	88.33	91	92.08	

Least Significance Difference (LSD) P<0.05, Isolate=0.9, Days=1.16, Isolate X Days=2.86, Coefficient of Variation (CV)=2.4

the toxin content also increases in the grain or rice based agricultural commodities.

The variations in the nutritional requirement of different isolates were also observed. On YGT medium the Isolate number 5, 9, and 11 had 71-80 mm colony diameter on sixth day. Whereas isolate number 13 and 20 had 60-70 mm colony diameter on same media and on the same day (Table-4). About 57 mm colony diameter of isolate number 8 was observed on YGT. On REA maximum growth i.e. above 50 mm colony diameter was recorded in isolate number 5, followed by 45-50 mm diameter in isolate number 8, 9, 11 and 13 (Table-3). Isolate number 20 had less than 40 mm colony diameter on REA whereas it had about 65 mm colony diameter on YGT.

The ITS region (Internal Transcribed Spacer) of ribosomal DNA was amplified and double digested with two restriction enzymes such as *EcoRI*, and *MspI*

Table-3

Periodic vegetative growth of isolates on Rice Extract Agar (REA) medium

Isolate No.	Colony diameter in Days of incubation									Mean diameter/isolate
	1	2	3	4	5	6	7	8	9	
AF-5	10.5	18.5	25.5	33.25	35.25	41.5	52.25	53.5	62	33.22
AF-8	6.25	11.5	17.5	31	39.75	43.25	50.5	55	59	31.37
AF-9	6	10.25	20.25	33	37.5	42.25	49	55	60	31.32
AF-11	6	11.5	18.75	31.5	37.25	42.5	46.5	51.5	55.75	30.12
AF-13	7	12.75	19.5	31.5	39.5	43.5	48.5	54	60	31.62
AF-20	7.5	13.5	17.5	23	30.75	36.25	38.5	42.75	46.5	25.62
Mean diameter/day	7.2	13	19.83	30.54	36.66	41.54	47.54	51.95	57.2	

Lowest Significance Difference (LSD) P<0.05; Isolate=0.97; Days=1.25; Isolate X Days, 3.07; Coefficient of Variation (CV) = 5.0

Table-4

Periodic vegetative growth of isolates on Yeast Extract Glucose Agar medium.

Isolate No.	Colony diameter in Days of incubation									Mean diameter/isolate
	1	2	3	4	5	6	7	8	9	
AF-5	18.75	32.25	47.75	53.5	59	67	76.75	83	88	52.6
AF-8	9.5	20.25	30	43.5	50	53	56.5	70.5	81.5	41.47
AF-9	9.75	21.25	33.5	46	52	57	75	81.25	85.75	46.15
AF-11	8.5	21.25	33.75	51.5	62.5	70.5	79	81	85	49.3
AF-13	9	24.25	33.5	49.5	54.5	58	61.5	64.5	74.75	42.95
AF-20	20.5	34	36.75	42	51.25	62	64.5	69.5	74	45.45
Mean diameter/day	12.66	25.54	35.87	47.66	54.87	61.25	68.87	74.95	81.5	

Lowest Significance Difference (LSD) P<0.05; Isolate =1.76; Days=2.27; Isolate X Days=5.57; Coefficient of Variation (CV) : 6

(*HpaII*). Banding patterns were visualized on agarose gel with Ethidium Bromide staining to observe molecular similarity and variation. It was found that in spite of significant variation in vegetative growth, isolates show molecular similarity with the protocol used in this study. The pattern of cultural growth on various media indicated the possibility of genetic variability among the isolates. However as it was not reflected in the amplified products, more sensitive techniques e.g. PAGE or AFLP may be required to study the genetic variation among the isolates.

Conclusion

Czapekdox Agar was found to be the ideal media for *A. flavus*. The test fungus preferred sucrose as carbon source. Yeast extract and micronutrient enhanced the growth. The variations in the nutritional requirement of different isolates were also observed. The growth pattern of isolates on Yeast extract Glucose Trace element media and Rice Extract Agar were different. For instance the isolate number 20 had less than 40 mm colony diameter on REA whereas it had about 65 mm colony diameter on YGT. However as genetic variability evident in growth pattern on different media was not reflected in the ITS region of ribosomal DNA, double digested with two restriction enzymes and banding patterns visualized on agarose gel with Ethidium Bromide staining. More sensitive techniques e.g. PAGE or AFLP with Silver Nitrate staining may be required to study the genetic variation among the isolates.

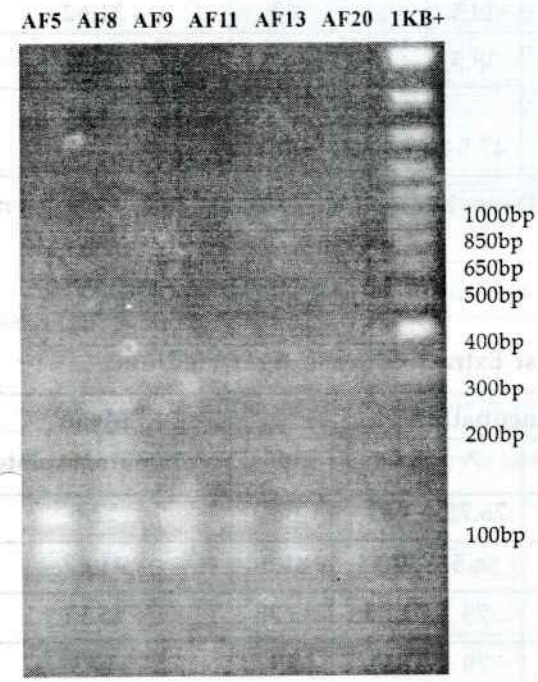

Figure-2: RFLP-ITS of ribosomal DNA shows molecular similarity among *Aspergillus flavus* isolates. PCR amplicons of ITS were double digested with *EcoRI* and *MspI (HpaI)* and visualized by agarose gel electrophoresis with Ethidium bromide staining (Mathiyazhagan *et al.*, 2006). Isolate no. in individual lane.

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Multiple shoot regeneration from cotyledonary embryonic axis explants of *Vigna mungo* (L.)

P.P. Behera, S. Dash, U. Mohapatra[1] and S.P. Rath

Department of Botany, Utkal University, Bhubaneswar - 75004

[1] Department of Botany, North Orissa University, Baripada -757003

Multiple shoots were induced efficiently from seed derived cotyledonary embryonic axis of Black Gram (*Vigna mungo* L.) cultured on MS medium supplemented with cytokinin. Presence of both the cotyledons either full or half resulted in maximum number of shoots. The regenerated shoots were elongated on MS basal medium containing benzylaminopurine (BAP) and rooted on MS medium containing indole-3-acetic acid (IAA). Rooted plantlets were transferred to soil successfully.

Key words: cotyledonary embryonic axis, shoot regeneration, *Vigna mungo*

Introduction

Grain legumes could adequately meet the protein requirements for a majority of population in India and black gram (*Vigna mungo* L. Hepper syn. *Phaseolus mungo Roxb.*) belonging to the Family Fabaceae (2n-22) is good source of dietry proteins. It is an important grain legume of the tropics and subtropics which provides an excellent source of easily digestible protein. The protein content in seed varies from 20-25%, carbohydrates 40-47%, lipids 1.3% and crude fiber 4.2% (AVRDC, 1975). Dried and green stalk and leaves of blackgram are used as fodder. Its nitrogen fixing ability increases nitrogen content of soil and hence improves fertility. Efforts to develop an efficient regeneration protocol from callus for the last two decades have so far yielded very little progress because of the recalcitrant nature of the plant. Plant regeneration in black gram was reported from shoot meristem (Bajaj and Dhanju, 1979; Goel *et al.*, 1983; Hoque *et al.*, 1984), cotyledons, embryos and embryonic axis (Gill *et al.*, 1987; Ignacimuthu *et al.*, 1997; Ignacimuthu and Franklin, 1999; Sen and Guha Mukherjee, 1998) ontogeny of somatic embryos (Eapen and George, 1990) and multiple shooting along with *in vitro* fruiting from cotyledonary nodes (Ignacimuthu *et al.*, 1997).

Teli *et al.* (2001) used MS medium for *in vitro* regeneration of *V. mungo* from different explants i e. shoot tips, cotyledons and embryonic axis culture of *V. mungo*. Sen & Guha Mukharjee (1998) used basal media B5 (Gamborg *et al.*, 1968) and MS (Murashige & Skoog, 1962) basal salts with B5 vitamins (MSB) for *in vitro* plant regeneration in *V. mungo* and two varieties of *V. radiata*.

In the present study a suitable protocol for obtaining plantlets from cotyledonary embryonic axis of *Vigna mungo* is derived.

Materials and Methods

Plant Material

Seeds of five varieties of *Vigna mungo* L., viz, PU35, Sarala and PU30 were collected from Center for Pulses Research, Orissa University of Agriculture & Technology, Bhubaneswar and PU19 and T9 were collected from ICAR, Kanpur, India in paper packets wrapped with polyethylene cover.

Surface sterilization

Seeds were kept under running tap water for 30 minutes followed by treatment with 5% (W/V) aqueous solution of Teepol (Reckitt's Cotman, Kolkata, India) for 8 minutes and rinsed 5 times with double distilled water. Then the seeds were surface disinfected with 0.1% (W/V) aqueous solution of mercuric chloride ($Hgcl_2$) (Hi media, Bombay) for 5 min followed by 5 rinses with autoclaved double distilled water.

Composition of basal media

MS (Murashige and Skoog, 1962) basal media was used supplemented with a range of plant growth regulators of different concentration and combinations. All media comprised a macronutrient and micronutrients sucrose (Hi-media, Bombay, India) 3% (w/v) as a carbon source and 0.8% (w/v) agar (Hi media, Bombay, India) as a gelling agent, amino acids and vitamins contents of all media are different.

Growth regulators

Growth regulators such as auxin and cytokinin used in present study were obtained from Sigma (USA). The individual growth regulators stocks were prepared by taking required amount of growth regulators in either ethanol or in KOH/NaOH as appropriate and were stored in amber bottles in laboratory refrigerator for future use. The growth regulators are added to medium at different final concentration before checking the pH. Cytokinin namely benzylaminopurine (BAP) Kinetin (Kn), thidiazuron (TDZ) were tested alone or in combination with auxins namely α-Napthalene acetic acid (NAA). For root induction auxins namely indole-3-acetic acid (IAA), indole-3- butyric acid (IBA) was tested alone or in combinations.

All operations including surface disinfection, inoculation and transfer of disinfected plant materials were conducted aseptically in a horizontal gradvel Laminar Air flow clean air work station (Klenzoids, India).

***In vitro* seed germination**

The surface disinfected seeds were placed on semisolid (gelled with 0.8% agar) half strength MS medium and full strength MS medium. The medium designated as ½ MS_o and MS_o was supplemented with 100 mgl^{-1} myo-inositol and 3% sucrose but lacked any growth regulator. Ten seeds were placed in each of the 100 ml conical flasks.

Explant inoculation

Five days old axencially grown seedlings were used as source of explants. After removal of radicle and primary shoot, the cotyledonary nodes of five genotypes were inoculated in 300 ml glass jars. Explants of different forms (Fig.1 A-D) such as cotyledonary node without cotyledon but with intact embryonic axis (Fig.1A), cotyledonary node with two proximal halves of cotyledon with embryonic axis (Fig.1B), cotyledonary node with single whole cotyledon with embryonic axis (Fig.1C) and cotyledonary node with two whole cotyledon with embryonic axis (Fig.1D) were inculcated on MS supplemented with 0.5-3 mgl^{-1} BAP. Here the different forms of cotyledonary nodes were taken to evaluate the effect of storage food present in cotyledon on shoot proliferation. The original explants were repeated three times subcultured on shoot multiplication medium (MS+2 mgl^{-1} BAP) after each harvesting of newly formed shoots. The primary shoots obtained were transferred to MS containing 2 mgl^{-1}BAP for axillary shoot proliferation.

Rooting of shoots

Well developed shoots of approx. 3.5-4.0 cm long were excised and transferred to 30ml culture tubes containing ½ MS and gelled with 0.8% (w/v) agar supplemented with 0.25-2 mgl^{-1} indole-3-acetic acid (IAA), indole-3-butyric acid (IBA). After seven days of root indication when roots were nearly 1 cm long, the rooted shoots were transformed to auxin free ½ strength MS for further elongation of roots.

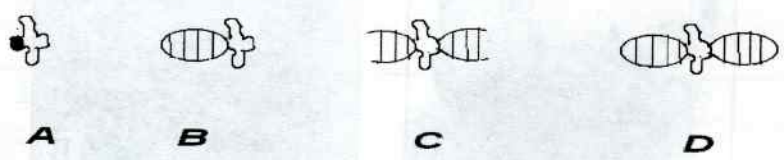

Fig. 1 (A-D) : Different forms of cotyledonary node explant. A. without cotyledons but with intact embryonic axis B. with single whole cotyledon with embryonic axis C. with two proximal halves of cotyledon with embryonic axis D. with two whole cotyledons with embryonic axis.

Acclimatization and transfer of plants to soil

Well rooted plantlets of seven days old were removed from culture medium and roots washed gently under running tap water to remove adhering agar. The plantlets were then transferred to plastic pots containing autoclaved vermicompost moistened with autoclaved water. The potted plants covered with polythene bags were maintained inside. After two weeks plants were transferred to clay pots containing garden soil and compost kept under shade for another week before transplanting to field.

Data recording, statistical analysis and photography

For shoot development experiments, each treatment consisted of ten replicates (culture vessels) and had two explants per vessel. In the rooting experiment, each treatment also consisted of ten replicates (culture tubes) and had one explant per tube. Each experiment was repeated thrice. Culture showing shoot differentiation, number of shoots per explant, shoot length, root number and root lengths were recorded after five days. Means of each treatment were calculated and the standard error was calculated. All information was recorded at seven days intervals.

Photographs were taken on Kodak colour films (Max 400) using SLR Camera Canon AE I program, Japan fitted with an auto extension tube (12 mm, 20mm, 30mm Triplus Japan) All colour prints were made in Colour Processing Laboratory (Unicolour Photolab, Bhubaneswar).

Results and Discussion

Media Evaluation for seed germination

MS medium either full or half strength without addition of growth regulators, was tested for seed germination efficiency in case of five different genotypes. Seeds started to germinate after three days. It was observed that in all cases ½ MS media was better responding than full strength media for seed germination (Plate 1 A-B). Genotype, Sarala responded best in which approximately 95% of seed *germinated on both MS and ½ MS medium.* Results of observation after five and ten days are given in Table-1.

Table-1

Seed germination efficiency in MS medium

Genotypes	Days	MS	½ MS
Sarala	5	75	92
	10	82	94
T9	5	70	82
	10	72	84
PU 30	5	68	70
	10	70	72
PU 35	5	61	63
	10	65	67
PU 19	5	60	62
	10	62	64

Data given are the mean percentage of seed germination.

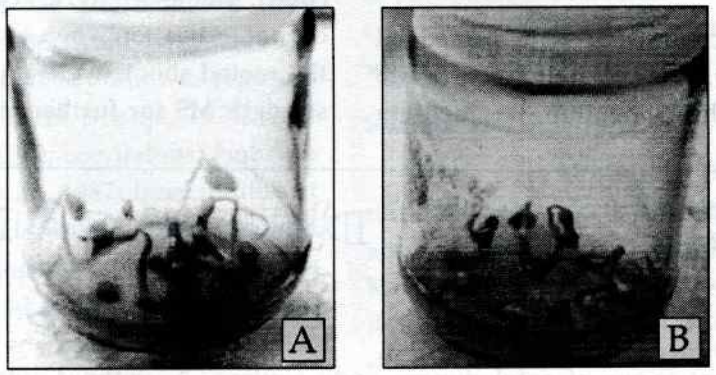

Plate 1: Efficiency of seed germination of *Vigna mungo* var Sarala in ½ MS_0 (A) and MS_0 (B) medium

Evaluation of shoot regeneration capability

The explants excised from five days old seedlings shows better response than ten days old seedlings. The growth regulator, cytokinin, was essential in the medium for regeneration of shoots from cotyledonary node explants. Of three cytokinins tested (BAP, Kn and TDZ), BAP was found most effective (Table-2). It was observed that addition of TDZ to the medium led to production of stunted and thickened shoots and TDZ was effective at lower concentration in comparison to BAP and Kn.

Five genotypes of blackgram could reveal an interesting result where shoots were regenerated in maximum up to 70% of the explants. In this case the medium was tested in MS+BAP medium and shoot proliferation started in the media containing BAP with minimum concentration of 0.6 mgl^{-1}. The percentage of explants for shoot regeneration increased with increase of concentration of BAP which was maximum at 2 mgl^{-1} and further increase of concentration of BAP decreased the shoot regeneration efficiency. So it was evaluated that the concentration and type of cytokinin was very much effective for the percentage of shoot regeneration, the number of shoots per explant and length of shoot. It was also found that the cytokinin (BAP) in combination with auxins (NAA) was less effective than single cytokinin (BAP).

Table - 2

Nodal explants of five varieties of *Vigna mungo* showing regeneration of shoots

Varieties	MS Medium		% Nodal explants with shoot regeneration	No. of shoots per explants
	BAP in mg/l	Kn in mg/l		
Sarala	1	-	90	5.2 ± 0.2
	-	1	50	4.8 ± 0.4
	-	2	80	6.1 ± 0.6
	2	-	97	7.0 ± 0.1
T9	1	-	60	4.6 ± 0.5
	-	1	45	3.8 ± 0.3
	-	2	66	5.5 ± 0.1
	2	-	75	5.8 ± 0.2
PU 30	1	-	61	5.6 ± 0.3
	-	1	58	4.3 ± 0.6
	-	2	75	5.9 ± 0.2
	2	-	80	6.2 ± 0.5
PU 35	1	-	70	4.0 ± 0.9
	-	1	60	3.6± 0.5
	-	2	71	4.0 ± 0.9
	2	-	82	6.3 ± 0.4
PU 19	1	-	76	2.6 ± 0.7
	-	1	32	2.9 ± 0.5
	-	2	50	3.4 ± 0.5
	2	-	83	4.9± 0.2

The values are the mean ± SE from 10 replicates.

Efficiency of different forms of cotyledonary node explants

Different forms of cotyledonary node explants with none, single or two full or half cotyledons attached to embryonic axis were tested for shoot regeneration efficiency (Fig. 1 A-D). It was seen that complete removal of both cotyledons caused delayed response or no response for shoot regeneration. Presence of cotyledon either full or half encouraged multiple shoots production (Plate 2 A-C). In MS with 2 mgl^{-1} BAP medium, explants shows maximum number of shoot regeneration in all five genotypes. Shoot regeneration efficiency of explants in all cases was markedly reduced when cotyledonary nodes were taken with only half of each cotyledon. The shoot regeneration efficiency decreased further when only one cotyledon was allowed with the cotyledonary node for culture. Shoot regeneration efficiency of the explants without any cotyledon shows very less response whereas well formed shoots arose from the explants with two full cotyledons containing embryonic axis indicating that the presence of full or part of cotyledon is essential for multiple shoot production. Complete removal of both cotyledons caused a delayed response of shoot regeneration and multiple shoots were not produced (Fig. 2).

Plate 2 A-C : Effect of number and size of attached cotyledons on multiple shoot regeneration of *Vigna mungo* var PU-19 in MS + 2mgl^{-1} BAP. A. Explant with single whole cotyledon after 7 days of culture. B. Explant with two proximal half of cotyledon after 15 days of culture. C. Explant with two whole cotyledon after 15 days of culture

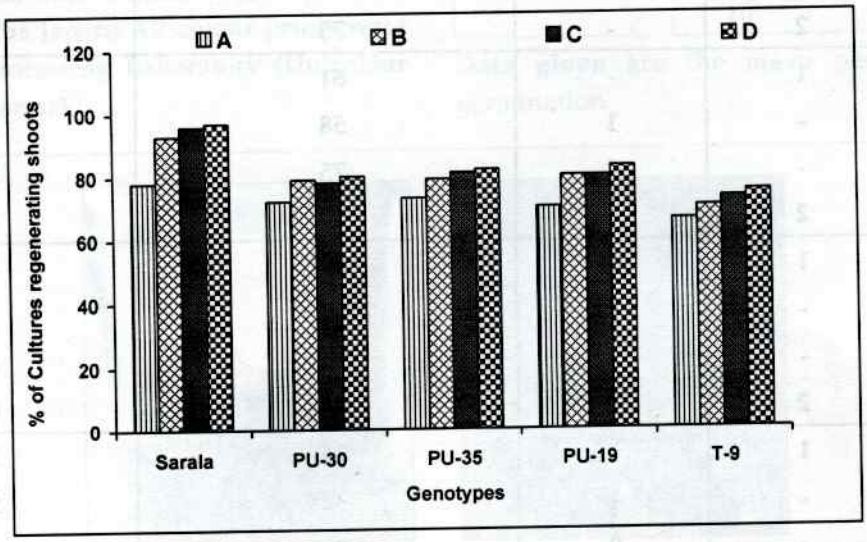

Fig. 2 : Effect of number of size of attached cotyledons on multiple shoot regeneration from cotyledonary node explants of *Vigna mungo* when cultured in MS+ 2 mgl^{-1} BAP. A-D refer to the different forms of cotyledonary node explant as per Fig. 1.

Evaluation of shoot regeneration efficiency of different genotypes

MS medium supplemented with 2 mgl^{-1} BAP was found to be more efficient for shoot regeneration from cotyledonary nodes of all genotypes. Out of five genotypes evaluated, Sarala, was the most responsive where from each explant an average of six shoots were developed and T9 was least responsive

Rooting of shoots

Rooting of in vitro regenerated shoots was achieved upon transfer to half strength MS medium supplemented with an auxin. For induction of root in excised shoots, ½ MS was better responsive than full MS. Auxin in the medium is important for rooting. ½ MS supplemented with IAA at 0.5 mg-l was found to be best suitable for rooting of excised shoots of all five genotypes within 10-15 days. From each shoot, an average 2-4 roots arose. *Vigna mungo* var Sarala showed better response in % of rooting, number of roots per shoot and root length in comparison to other genotypes after 15 days of inoculation in ½ MS + 0.5 mgl^{-1} IAA (Fig. 3).

Soil Establishment of *in vitro* grown plants

Well rooted plants were transferred to soil and kept covered with polythene bags to maintain humidity and later transferred to pots. Out of four different types of soil i.e. vermiculite, soiltritemix, vermicompost and garden soil, vermicompost was found more suitable (Table-3). After the acclimatization of plants in vermicompost the plants are transferred to the garden soil successfully (Plate 3 A-C). Out of five different varities studied *V. mungo* var Sarala shows better survival (78%) and variety T-9 shows least survival (56%).

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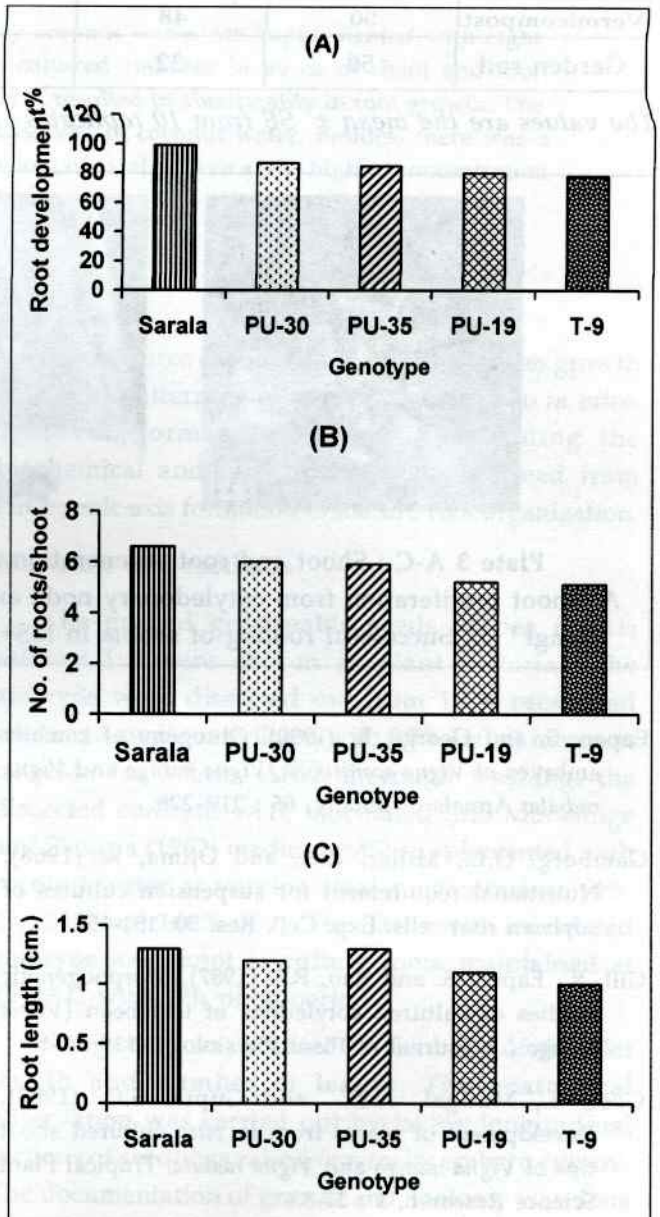

Fig. 3 : Genotypic effect on % of rooting (A), number of roots per shoot (B) and root length in cm (C) during root development from *in vitro* derived shoots after 15 days of inoculation in ½ MS + 0.5 mgl^{-1} IAA in *Vigna mungo*.

Table - 3

Evaluation of different planting substrate for acclimatization of *in vitro* explants of *V. mungo* var Sarala

Plants substrate	No. of Plants transferred	No. of Plants survived	Survival %	Shoot height in cm.	No. of leaves/ plantlets	Root length in cm.
Soilritemix	50	42	84	10.4 ± 1.7	4.6 ± 0.3	5.2 ± 0.1
Vermiculite	50	33	66	7.8 ± 1.1	3.8 ± 0.2	4.1 ± 0.3
Vermicompost	50	48	96	16.9 ± 1.8	7.6 ± 0.4	7.1 ± 0.2
Garden soil	50	32	64	5.1 ± 1.45	3.8 ± 7.2	3.3 ± 0.1

The values are the mean ± SE from 10 replicates

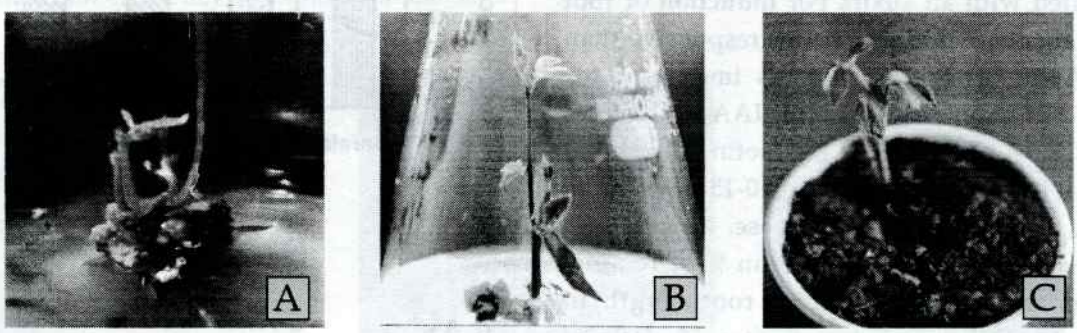

Plate 3 A-C : Shoot and root regeneration and aclimatization in *Vigna mungo* var Sarala. A. Shoot proliferation from cotyledonary node explants of from single whole cotyledon in MS +BAP 2mgl^{-1} B. Successful rooting of shoots in MS+IAA0.5 mgl^{-1} C. Aclimatization in field condition

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Change in axial pattern of *in vitro* seedlings from embryo cultures of *Pisum sativum* L.

A.K. Bhadra, A.K. Dalai and B. Behera
Department of Botany, Ravenshaw University, Cuttack-753 003, Orissa

In vitro growth of pea embryos was influenced by coconut water. MS supplemented with eight per cent coconut water produce a positive effect on cultured embryos in terms of shoot and root growth. Higher concentration of coconut water (20% v/v) resulted in abnormality in root growth. The root system was completely inhibited in MS salts dissolved in coconut water. Besides, there was a tendency of hypocotyl region to become tuberous. The loss of axial pattern at the highest concentration of coconut water is attributed to its effect on vasculature.

Key words: Axial pattern, coconut water, vasculature

Introduction

The study of biological science centers on exploitation of phenomena of growth and development. There is a need to develop model for explaining the phenomenon of differentiation at molecular level (Hall, 1973).

Differentiation can be studied in two directions: the actual process of differentiation and the state of being differentiated. The dicot embryo has the embryonic axis showing a distinct axial pattern. Following germination the embryonic axis starts growing with maintenance of the pattern in the resulting seedling.

The root tip of developing seedlings is the site to generate cytokinin like substance that influences shoot morphogenesis (Hall, 1973). The apical region of the shoot is the site of synthesis of auxin.

Exogenous growth regulators can be used as a probe to study the complexity of morphogenesis (Hall, 1973). The root morphogenesis is a suitable process for study in comparison with that of shoots due to a simpler radial pattern of the former. Besides, roots are usually quite sensitive to applied hormones (Torrey, 1976).

Keeping in view the role of exogenous growth regulators on morphogenesis the present investigation aims to study the effect of coconut water, generally used as a source of plant growth regulator, on growth and axial patterning of pea embryos grown *in vitro*. This will form a basis for understanding the biochemical and molecular events that lead from embryonic axis formation to mature root organization.

Materials and methods

The graded germinable seeds of pea (*Pisum sativum* L.) were chosen as plant material. The embryos were dissected out from 18 h presoaked seeds and surface sterilized with 0.1% (w/v) mercuric chloride for 3 min. After thorough washing, the dissected embryos were inoculated into Murashige and Skoog's (1962) medium (MS) supplemented with coconut water at varying final concentrations (4%, 8%, 20% and 100% v/v). The flasks with inoculated embryos were kept in culture room maintained at 25±1° C with 12h photoperiod.

The growth was studied on the basis of shootlet length and number of leaves. The anatomical observation was carried out by taking longitudinal sections of seedlings raised *in vitro* by embryo culture. The documentation of growth and anatomy was done through digital photography.

Results

In vitro growth of pea embryo was found to show variation with different concentrations of coconut water supplemented to MS medium (Fig. 1). There

was an increase in the seedling growth with increase of concentration of coconut water (up to 8%) on 20th day of observation after inoculation. Fig. 2 depicts the well-differentiated growth of seedlings with root and shoot developed from embryo grown in media supplemented with 8% coconut water. The proportion of growth of root and shoot in MS supplemented with 4% and 8% coconut water is depicted in Fig. 6.

With higher concentration of coconut water there was abnormality in root growth though shoot growth was found to remain more or less unaffected (Fig. 3 & 4). Twenty percent coconut water supplement was found to cause abnormality in root growth. In some cases there were swollen hypocotyls with feeble root system (Fig. 3) whilst in others there were swollen hypocotyls with calli but without roots (Fig. 4).

The most remarkable result was the swollen hypocotyl with complete absence of root and stunted shoot growth (Fig. 5 & 7) in MS medium made of coconut water (100% v/v). The swollen region was like an elongated tuber.

The anatomy of normal pea plant at the epicotyl and hypocotyl region is shown in Fig. 8. The longitudinal section of the region depicts well continuity of vasculature from root to shoot. The vascular axis was also found to have the connection with cotyledon through lateral vasculature from the axis. However, the longitudinal section of the abnormal pea plant (Fig. 9) developed through *in vitro* culture of embryo on MS with only coconut water (100% v/v) was found to show abnormality in the vascular axis. Towards the root region from the cotyledonary node there was discontinuity of vascular axis (Fig. 9, arrow mark). The section taken through tuber like growth depicts the arrangement of vascular bundles at the medullary region (Fig. 10 A). The peripheral region of the section of tuber was found to have suberised cells (Fig. 10 B).

Discussion

Cytokinin is commonly used in tissue culture as it promotes cell division. Coconut water is often used in embryo culture and seedling development due to its high nutritional status and growth regulating activity. Coconut water can be considered as a source of cytokinin (Kobayashi *et al.*, 1995). In the present investigation coconut water was used to consider its effect on pea embryo culture. The lower concentrations of coconut water (up to 8%) were found to promote root and shoot growth. It can be considered that the coconut water is a source of nutrients and growth regulators, which at lower concentration promotes the growth of embryos and their conversion to seedlings. The abnormality of seedling growth was marked at higher concentration

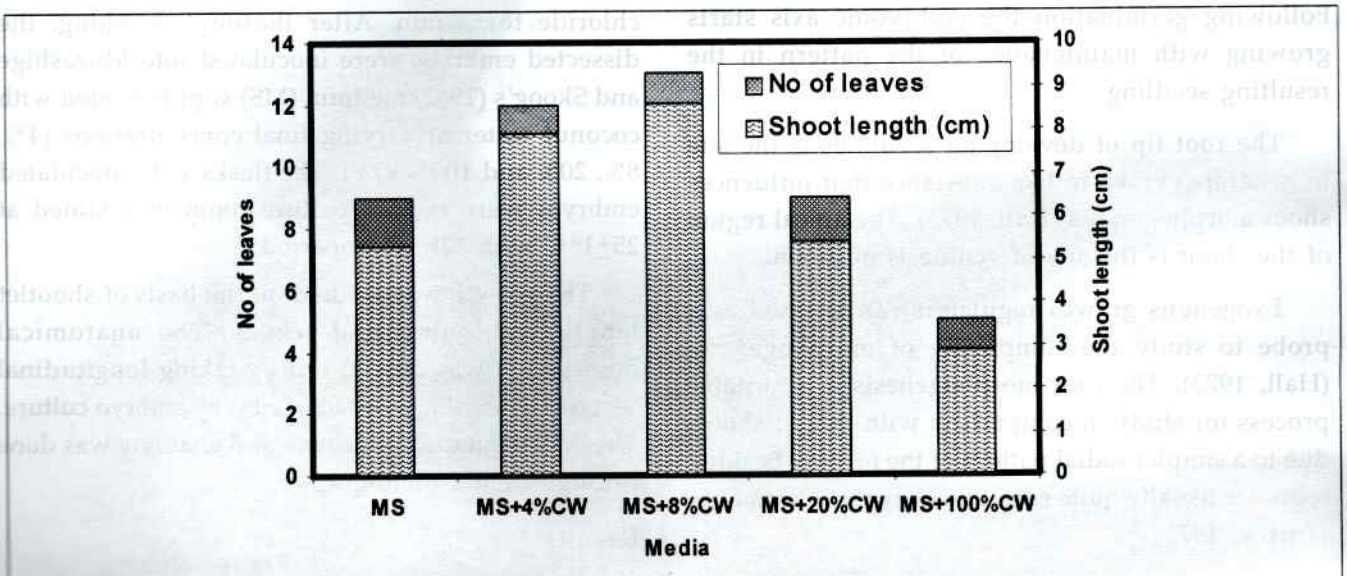


Fig.1 Growth of in vitro cultured pea embryos in MS media supplemented with different concentration of coconut water

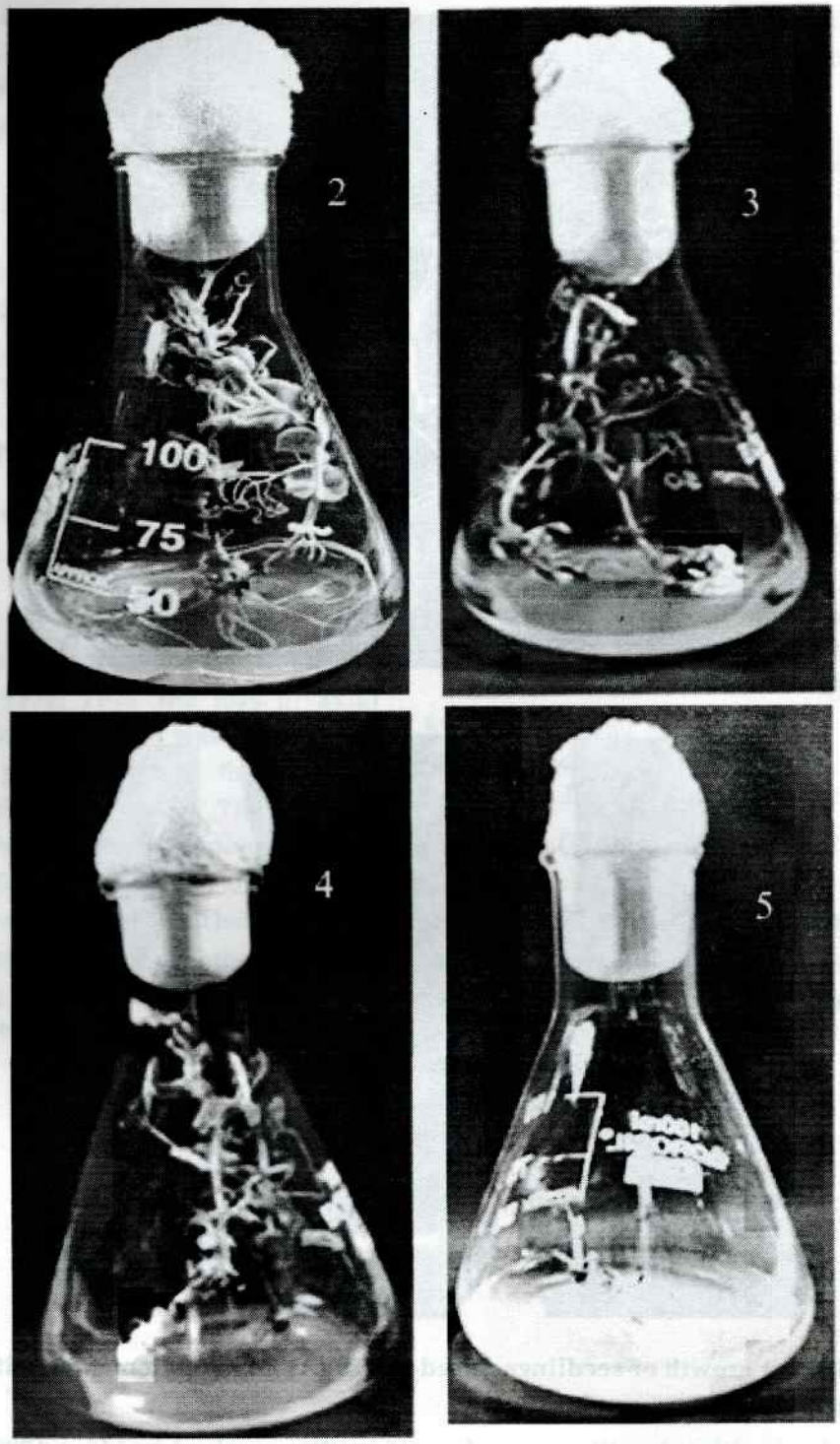
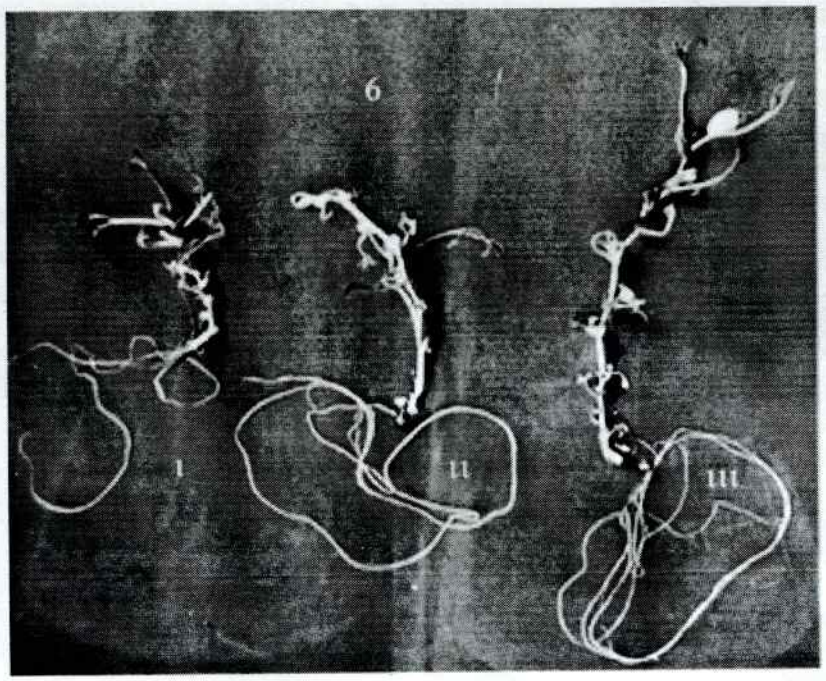


Fig. 2 : Seedlings raised in MS + 8% coconut water.

Fig. 3 : Seedlings raised in MS + 20% coconut water having swollen hypocotyl and reduced root growth.

Fig. 4 : Seedlings raised in MS + 20% coconut water having calli at hypocotyl and without root.

Fig. 5 : Seedlings raised in MS + 100% coconut water having stunted shoot without root.



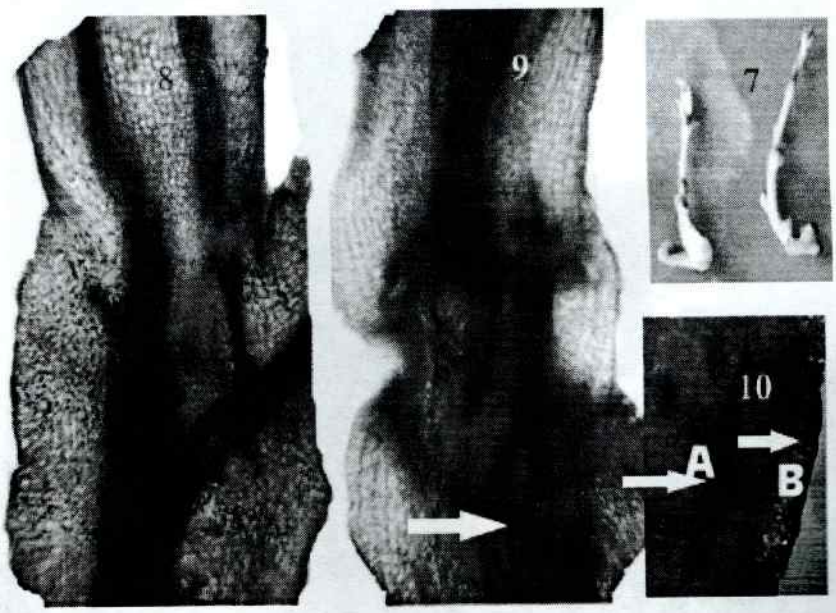


Fig. 6 : Root and shoot growth of seedlings raised in MS (A), MS supplemented with 4% (B) and 8% (C) coconut water.

Fig. 7 : Stunted shoot with tuber like appendage of seedlings raised in MS + 100% coconut water.

Fig. 8 : Longitudinal section of normal seedling showing continuation of vasculature.

Fig. 9 : Longitudinal section of abnormal seedling showing discontinuation of vasculature (arrow mark)

Fig. 10 : Transverse section of tuber below cotyledon showing medullary vasculature (A) and outermost layer of suberised cells (B)

of coconut water (20% and 100%). The maximum abnormality was with respect to root growth and development. The development and architecture of plant roots are regulated by phytohormone (Torrey, 1976); especially the cytokinin which when applied alone can cause a net growth inhibition in roots (Scott, 1972). The results of the present investigation corroborate this general finding. The swelling of hypocotyledonary region to form tuber like structures under coconut water treatment confirms the finding of Skoog and Armstrong (1970) who demonstrated the inhibition of root growth and induction of tuber formation resulting from cytokinin treatment.

The axial pattern of the pea embryo during its growth was found to be disrupted under the treatment of coconut water as was supported by the anatomical findings. The organized differentiation of vascular tissue is known to be influenced by cytokinin (Aloni, 1993). Thus the loss of axial patterning at the vascular level as was noted in the present study was perhaps due to the effect of supraoptimal level of coconut water. The anatomy of the swollen hypocotyledonary region was also found to show the medullary vascular bundles and suberised cells at the outer region. These are also the characters of tuber, which confirm the finding of Skoog and Armstrong (1970). The abnormality of root growth as an observation of the present investigation can form a basis for understanding the molecular events that lead to axial patterning of seedlings.

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Study on the impact of Sugar Mills Effluents on germination behaviour and some biochemical *parameters of two crop plants, Cajanas cajan L.* (Red gram) and *Triticum aestivum*, L. (Wheat)

Sibani Patro[1], Sradhanjali Sahu[1], P. Sudhakar[1], U.K. Sahu[2] and B.K. Mohanty[1]

[1]PG Department of Botany & Biotechnology
Khallikote Autonomous College, Brahmapur - 760 001, Orissa, India
[2]SKCG Autonomous College, Paralakhemundi-761 200, Gajapati, Orissa, India

Effect of Sugar Mill effluents on Wheat and Red gram seedlings was studied. The effluent significantly inhibited germination root and shoot length in both the crop plants. The biochemical injury does not appear spontaneously, but with the increase in effluent treatment there is reduction in observed biochemical parameters (Chlorophyll, Protein, Amino Acid, Nucleic acids and Carbohydrate) which was negatively correlated. The shoots of the seedlings were found to be resistant, where as root of the seedling were susceptible to sugar mill effluent in both the cases. The dilution of effluent has stimulated the seedling growth and all observed biochemical parameters. The effect of the sugar mill effluents was more pronounced in Wheat than in Red gram seedlings.

Key words : Red gram, Wheat, effluent, germination, seedlings.

Introduction

The harmful effect of sugar mill effluents has been well documented. Most of the studies have been carried out to find out the effect of effluent on the crop plants (Kadioglu and Algur, 1990; Tiwari *et al.*, 1993; Muthu Kumar and Arockisamy, 1994 and Subramani *et al.*, 1995). The studies of Pragasam and Kannabiran (2001), Kannabiran and Pragasam (1993, 2001) are related to the effect of distillery effluent on reading characteristics of *Vigna mungo* which reported that low concentration of Distillery effluent may have a beneficial effect on the seedlings. Studies by other authors (Pragasam and Kannibiran, 2001; Indria and Mohanty, 2006; Mahapatro and Mohanty, 2007) revealed that the distillery effluent had stimulated the plant growth at lower concentration with high concentration inhibiting it. Most of the studies were concerned with the seed germination and seedling growth only. The present work brings out the effect of sugar mill effluent on the growth behaviour of seedlings and all the important biochemical parameters to correlate the events.

Materials and methods

Aska Co-operative Sugar mills situated in the town of Aska (Orissa) which discharges the factory effluent into land or nearby water bodies which ultimately reaches the River Rushikulya and is perennial source of water for domestic use and irrigation of crop land for the people residing village along the river course. The industrial effluent generated by from ACSI, Aska used for the present investigation. The physico-chemical characteristics of ACSI Aska are given in Table-1 and 2. The factory claims to have undertaken all required effluent treatment measures to reduce the pollutant load in the waste water/effluents generated.

For our experimentation, the Sugar Mill effluent was collected from the source at the point of discharge. The collected effluent was stored at 10°C and was brought to room temperature before they are used in experimentation. The collected effluent was considered as stock (100% concentration) and diluted to 5, 10, 15, 20, 25, 50, 75, 100% (volume/

Table-1

Characteristics of Sugar Mills Waste

pH	6.7
COD, mg/I	3500 - 4000
BOD, mg/I	1600 - 2000
Total Solid, mg/I	1050 - 3500
Total Volatile, mg/I	480 - 2200
Suspended Solids, mg/I	400 - 800
Total Nitrogen, mg/I	10 - 40
BOD/COD ratio	1.6 - 2.0

[N.B.: The data are of 5 (five) years average for ACSI, Aska]

Table-2

Analysis of Spent Wash from ACSI Distillery

Volume I/I of rectified spirit	20
Color	Dark Brown
Odor	Burnt Sugar
Temperature	94 - 98^{0}C
pH	3.7 - 4.0
BODS 20^0 C mg/I	52000 - 59000
COD mg/I	126000 - 148000
Total solids mg/I	94000 - 106000
Total dissolved solids	93000 - 98000
Total volatile solids mg/I	81000 - 91000
Total dissolved solids (inorganic) mg/I	10500
Suspended solids (inorganic) mg/I	1000 - 2000
Total Nitrogen mg/I	2300
Total phosphorous (p) mg/I	441
Potassium mg/I	4280
Calcium mg/I	80
Sodium mg/I	250
Sulphate mg/I	1207
Chlorides mg/I	1920

volume) with distilled water for experimentation. A set with distilled water were also maintained and taken as control.

Healthy seed of Red gram (*Cajanas cajan*) and Wheat (*Triticum aestivum*, L.) was obtained from O.U.A.T. extension centre, Ratnapur, Berhampur, Ganjam, and was preserved in an incubator at 19°C. The seeds were graded and surface sterilized with 1% $Hgcl_2$ before use.

Germination beds were prepared taking sterilized cotton and blotting paper in each sterilized Petri plate (6''dia) 30 nos. of seeds were taken in each set and were treated with effluents of desired concentration. Emergence of radical was considered as the index of germination.

Roots and shoots of 7 day old plant were taken for estimation of various biochemical parameters. Chlorophyll contents was estimated following the procedure of Arnon (1949). Chlorophyll was extracted by grinding the leaf material from shoot in a mortar and pestle with 80% Acetone(V/V). Homogenate was centrifuged and supernatant was collected in a test tube. To the residue again 80% Acetone was added, stirred and centrifuged. Both the supernatant were pooled together into a convenient volume and Absorbance was noted at 645 and 653 nm against Acetone blank by a spectrophotometer. The total chlorophyll content was calculated and expressed in terms of mg/g fresh tissue.

The pre-weighted shoot or root sample was grinded in a mortar and pestle with 70% Alcohol (V/V). The homogenate was centrifuged and supernatant was collected. To the residue, 70% alcohol was added, centrifuged and supernatant was collected. Both the supernatant were pooled together to estimate the Amino acid content by the method of Moore and Stein (1948) using L-leucine as standard.

5ml of 2N NaoH was added to the residue collected from the above and the tube were kept at room temperature for 1hour. Then they were kept in boiling water bath for 20 minutes, cooled and centrifuged. The supernatant were collected for Protein estimation following the method of Lowry *et al.* (1957) using BSA as standard.

The pre-weighed shoot and root samples were taken and was grinded with 70% ethanol and centrifuged. To the residue, 70% hot ethanol was added and again centrifuged. To the residue, 10% TCA was added and refrigerated overnight. To the collected residue, 5ml 10% TCA was added and kept in a boiling water bath for 10-20 minutes, cooled and centrifuged. The supernatant was used to estimate the nucleic acid content (DNA and RNA) following the method of Schmeider (1957).

The Soluble Sugar was extracted from the shoot and root samples by grinding pre-weighted material in 70% alcohol, centrifuged and supernatant was discarded. The process was repeated again and to the residue, 10% TCA was added, kept in a boiling water bath for evaporation of alcohol till 1-2 ml left in the tube. After making the volume to convenient level the soluble sugar was estimated using Anthrone reagent (Yoshida *et al.*, 1972).

All the data were expressed in terms of mean ± SD of ten replicates. The correlation analysis was carried out between percentage of effluent concentration and parameter studied and correlation coefficient values are expressed in terms of (r) value at various levels of significance (P) (Mishra and Mishra, 1989).

The germination of seeds were recorded after 72 hours of experiment setting both in control and effluent treated seedlings. In general there is decrease in germination as one of the important manifestations of increase concentration of effluent. Percentage germination of seeds was reduced and became 27.7% (Red gram) and 11.08% (Wheat) in case of 100% effluent concentration i.e. undiluted effluent (Table-3).

Red gram and Wheat seeds took about 72 hours for total germination and again 72 hours allowed for gradual growth of radicle and plumule. Thus root and shoot length measured at end of 7th day. By this time seeding have their first pair of leaves.

Vegetative growth of the seedling were measured and ratio was calculated between the root and shoot length under varied concentration of effluent (Table-4). It shows that root and shoot length decreased with increase in effluent concentration and in both the case it was negatively correlated with higher statistically significance level in the same manner the root and shoot ratio also decreased from control with increase in the effluent concentration (Table-4).

Table 5 and 6 deceipt the results observed in relation to various biochemical parameters like chlorophyll, Amino Acids, protein, DNA, RNA and Carbohydrates in root and shoot with treatment of sugar mills effluents with varied concentration (5% to 100%) to both the cultivars. The biochemical parameters were slightly increased with the increase in concentration of effluent treatment. When the effluent concentration was increased (50, 75 & 100%), there was a decrease in biochemical parameters studied, which showed a negative correlation. The shoots are more affected than roots incase of DNA and RNA studies in Red gram seedlings. The Wheat seedlings were comparatively more affected in biochemical parameters.

Discussion

Seed germination and growth are of vital importance for continuation of plant life. For

Table-3

Effect of different concentrations of effluent of Germination percentage of Red gram and Wheat seeds (Germination percentage was noted after 72 hours of soaking)

Cone of Effluent (%) V/V	% of Seed Germination ± SD	
	RED GRAM	WHEAT
0	97.22 ± 4.81	94.44 ± 5.56
5	91.66 ± 8.33	79.94 ± 5.5
10	74.99 ± 5.32	79.6 ± 6.0
15	69.44 ± 9.62	66.6 ± 8.2
20	66.6 ± 8.33	61.6 ± 5.2
5	61.6 ± 4.8	62.96 ± 4.3
50	49.9 ± 2.2	49.5 ± 5.5
75	41.6 ± 1.9	41.7 ± 3.17
100	27.7 ± 3.4	11.08 ± 5.52

germination and seeding growth entrance of water into seeds is a basic requirement to initiate and trigger the intricate sequence of metabolism. At this stage the seed and seedlings are susceptible to environmental stress like the presence of pollutants (ex-effluents) in the environment of germinating seeds hence resulted in deleterious effect on germinating seeds and seeding growth.

A number of studies have been carried out to find out the effect of the effluents of distillery on crop plants Kadioglu and Algur (1990), Tiwari et al., (1993). Muthukumar and Arockiasamy (1994) Subramani et al., (1995) and the effect of distillery effluent on various aspects of red gram and black gram has also been reported (Kannabiran and Pragasam. (1993) Pragassum and Kannabiram (2001,) Attempts was also made in past to assess the effect of distillery effluents on rice seedlings by Sahai et al., (1983) on *Cajanus cajan,* Sahai and Shrivastava (1986) and on *Helianthus annus* by Rajendran (1990) found to be invariable beneficial up to 5 % of the effluent concentration.

There was retardation in seeding growth and this case can be attributed to the impact of sugar mill effluents [Pragassam and Kannabiran (2001) Sharma et al., (2002) and Kanan (2001)]. The retardation the root growth in the present case inferred in term of root length in control and effluent treated seedlings suggests that there is high oxygen demand of the sugar mill effluent (Table 1&2). This was also in

Table-4

Effect of different concentrations of sugar mill effluent on root and shoot length of 7 day old red gram and Wheat seedlings. Each datum is represented as mean ± S.D.

% of Effluent Conc.	Type of Seedling	Mean ± SD Root Length (cm)	Mean ± SD Shoot Length (cm)	R/S
0	Red gram	13.5 ± 2.99	7.83 ± 1.06	0.58
	Wheat	15.5 ± 4.23	10.08 ± 2.46	1.53
5	Red gram	14.7 ± 2.88	7.85 ± 1.59	0.53
	Wheat	14.66 ± 1.08	14.9 ± 1.87	0.98
10	Red gram	11.66 ± 2.819	6.75 ± 1.20	0.57
	Wheat	14.01 ± 4.74	8.5 ± 1.87	1.64
15	Red gram	8.8 ± 1.109	7.5 ± 1.10	0.85
	Wheat	13.75 ± 2.36	8.16 ± 2.78	1.67
20	Red gram	8.58 ± 1.22	6.76 ± 1.62	0.71
	Wheat	10.25 ± 6.24	7.33 ± 2.16	1.39
25	Red gram	11.4 ± 1.264	6.31 ± 0.62	0.55
	Wheat	8.85 ± 5.43	5.66 ± 1.08	1.56
50	Red gram	6.45 ± 0.74	8.15 ± 0.73	1.26
	Wheat	6.5 ± 3.74	4.91 ± 1.39	1.32
75	Red gram	6.81 ± 0.17	6.21 ± 0.29	0.91
	Wheat	5.2 ± 1.3	4.02 ± 0.87	1.29
100	Red gram	4.2 ± 0.7	4.58 ± 0.9	1.09
	Wheat	2.3 ± 0.7	1.5 ± 0.4	1.53

Table-5

Effect of Sugar Mill effluents on root and shoot of 7 day old *Triticum aestivum* seedlings on various biochemical parameters. (Figures in parenthesis shows the percentage in biochemical parameters from control values)

Effluent Treatment		Control %	Effluent 5%	Effluent 10%	Effluent 15%	Effluent 5%	Effluent 20%	Effluent 25%	Effluent 50%	Effluent 100%	Correlation coefficient with level of significance
Chlorophyll Content mg./g fr. wt.		13.82	15.56 (12.59)	12.85 (-7.01)	12.66 (-8.39)	12.79 (-7.45)	12.11 (-12.37)	11.52 (-16.64)	10.78 (-21.99)	9.87 (28.58)	0.223 (P; NS)
Protein Content (mg/g.)	Shoot	16.15	20.28 (+25.57)	16.15 (0)	9.26 (-42.66)	8.46 (-47.61)	7.53 (-53.37)	6.82 (-57.77)	4.38 (-72.87)	3.33 (-79.38)	0.712 (P; NS)
	Root	6.66	15.12 (+127.02)	14.35 (+115.46)	8.93 (+34.08)	5.05 (-24.17)	4.99 (-25.07)	3.88 (-41.74)	3.12 (-53.15)	2.28 (-65.76)	0.623 (P; NS)
Carbohydrate content (mg./g)	Shoot	9.73	15.11 (+55.29)	11.42 (+17.36)	10.56 (+8.53)	9.05 (-6.98)	8.07 (-17.06)	8.03 (-17.47)	5.67 (-41.72)	5.79 (-40.49)	0.660 (P; NS)
	Root	2.89	9.66 (+234.25)	9.39 (+224.91)	7.67 (+163.32)	7.79 (+169.55)	5.02 (+73.70)	4.35 (+50.57)	3.38 (+16.95)	2.76 (-4.49)	0.530 (P; NS)
Amino Acid content (mg/g)	Shoot	10.28	12.21 (+18.77)	10.56 (+2.72)	10.11 (-1.65)	9.37 (-8.85)	8.28 (-19.45)	5.53 (-46.20)	4.31 (-58.07)	3.91 (-61.96)	0.819 (P£0.05)
	Root	11.42	7.8 (-31.69)	8.6 (-24.69)	8.6 (-40.45)	5.46 (-52.18)	4.25 (-62.78)	3.82 (-66.54)	3.65 (-68.03)	3.32 (-70.92)	0.683 (P; NS)
DNA Content (mg/g.)	Shoot	12.4	13.9 (+12.09)	10.8 (-12.90)	11.5 (-7.25)	9.07 (-26.85)	8.1 (-34.67)	5.03 (-59.43)	4.67 (-62.3)	4.53 (-63.46)	0.782 (P; NS)
	Root	9.8	8.1 (-17.34)	7.45 (-23.97)	5.2 (-46.93)	4.5 (-54.08)	4.16 (-57.55)	4.25 (-56.63)	3.81 (-60.30)	3.04 (-68.97)	0.658 (P; NS)
DNA Content (mg/g.)	Shoot	14.2	9.9 (-30.28)	10.2 (-28.16)	8.6 (-39.43)	6.25 (-55.98)	8.23 (-42.04)	5.42 (-61.83)	4.9 (-65.49)	4.61 (-67.53)	0.695 (P; NS)
	Root	9.27	8.31 (-10.35)	7.2 (-22.3)	6.77 (-26.96)	5.41 (-41.63)	4.82 (-48.00)	3.61 (-61.05)	2.85 (-69.25)	2.36 (-74.54)	0.801 (P≤ 0.05)

Values are in mean ± SD of ten samples, correlation coefficient (r) values are calculated between concentration or effluent treatment and different parameters studies. Significance levels (p values) are shown in parentheses.

NS: Not Significant

Table-6

Effects of sugar effluents on root and shoot of 7 days old *Cajanas cajan* seedlings on various biochemical parameters (Figures in parentheses shows the percentage in-biochemical parameters from control values.)

Effluent Treatment		Control %	Effluent 5%	Effluent 10%	Effluent 15%	Effluent 5%	Effluent 20%	Effluent 25%	Effluent 50%	Effluent 100%	Correlation coefficient with level of significance
Chlorophyll Content mg./g fr.wt.	Leaves	11.88	15.36 (+29.29)	14.01 (+17.92)	13.62 (+14.64)	10.59 (-10.85)	10.02 (-15.65)	9.56 (-18.77)	9.2 (-22.55)	8.66 (-27.60)	0.582 (P; NS)
Protein Content (mg/g.)	Shoot	24.5	29.9 (+22.40)	24.5 (0)	19.5 (-20.40)	18.5 (-24.48)	16.4 (-33.06)	14.5 (-40.8)	12.5 (-48.97)	11.3 (-53.87)	0.72 (P;NS)
	Root	19.9	21.15 (+6.28)	20.85 (+4.77)	19.4 (-2.51)	13.4 (-32.66)	12.46 (-37.38)	11.8 (-40.7)	10.45 (-47.48)	9.58 (-51.85)	0.704 (P;NS)
Carbohydrate content (mg./g)	Shoot	13.04	14.84 (+13.8)	14.48 (+11.04)	14.02 (+7.51)	12.78 (-1.99)	12.58 (-3.52)	11.4 (-12.57)	10.6 (-18.71)	9.32 (-28.52)	0.662 (P; NS)
	Root	23.96	18.54 (-22.62)	15.28 (-36.22)	13.6 (-43.23)	13.82 (-42.32)	12.3 (-48.66)	11.08 (-53.75)	8.16 (-65.94)	7.52 (-68.61)	0.722 (P; NS)
Amino Acid content (mg/g)	Shoot	13.66	14.4 (+5.41)	14.07 (+3.00)	12.43 (-9.04)	12.47 (-8.71)	10.98 (-19.61)	11.86 (-13.17)	10.66 (-21.66)	9.87 (-27.74)	0.565 (P; NS)
	Root	17.22	21.91 (+27.23)	18.91 (+9.81)	18.01 (+4.58)	18.75 (+8.88)	16.68 (-3.13)	13.5 (-21.6)	12.4 (-27.99)	10.82 (-37.16)	0.718 (P; NS)
DNA content (mg/g)	Shoot	6.3	6.9 (+8.52)	7.4 (17.46)	5.4 (-1.42)	4.4 (-30.15)	3.1 (-50.79)	2.9 (-53.96)	2.2 (-65.07)	1.8 (-71.42)	0.762 (P≤0.05)
	Root	5.9	5.3 (-10.16)	4.85 (-17.79)	4.1 (-33.89)	3.9 (-33.89)	3.7 (-37.28)	3.2 (-47.11)	3.82 (-35.25)	2.45 (-58.47)	0.743 (P≤0.05)
DNA content (mg/g)	Shoot	12.3	14.5 (+17.88)	13.2 (-7.31)	12.7 (+3.28)	9.8 (-20.32)	7.5 (-39.02)	5.8 (-52.84)	6.2 (-49.59)	9.8 (-60.97)	0.750 (P≤0.05)
	Root	10.8	11.6 (+7.40)	11.05 (+2.31)	8.4 (-22.22)	7.5 (-30.55)	7.64 (-34.81)	6.5 (-39.81)	5.78 (-46.48)	5.20 (-51.85)	0.703 (P;NS)

Values are in mean ± SD of ten samples, correlation coefficient (r) values are calculated between concentration of effluent treatment and different parameters studies. Significance levels (p values) are shown in parentheses.

NS : Not significant

agreement with the reports of Swaminathan and Vaidheswaran (1992) on groundnut seeds in oxygen depleted environment. The inhibition of germination and reduction in root and shoot length also in *Elusine caracana* reported by Mishra *et al.*, (1989) was attributed to the mito-depressive activity of sugar mill effluents.

It is now commonly believed that effect of pollutants like effluents occur at biochemical level, which finally results in delay in germination and retardation in seedling growth. However biochemical injury in seedling does not appear spontaneously. When the concentration of pollutant effluents exceed the detoxifying capacity of the tissue through their normal metabolism there is decreases in the biochemical parameters (like chlorophyll) protein amino acid sugar and nucleic acids) which is again directly proportional to the concentration of effluents.

A decreases in chlorophyll content in effluent treated seedlings suggests pollution injury (Pragassam and Kannabiran, 2004) changes in pigment concentration by sugar mill effluent treatment (Sharma *et al.*, 2002) also likely to affect carbohydrate metabolism, hence, decrease in chlorophyll content supports the above view.

The increases or decreases in the amount of metabolites either may be due to decrease synthesis or increased metabolism and chemical destruction of metabolite itself (Panigrahy, 1996). The reductions in biochemical parameters in effluent treated seedling of Red gram and Wheat seedlings in the presence of effluent indicate impairment of metabolic machinery.

In all biochemical parameters studies (Chlorophyll, amino acid carbohydrate DNA and RNA) the shoot of seedling was comparatively resistant whereas root was susceptible to the effluent treatment.

The biotoxic effect of the effluents of ACSI. Aska on Red gram and Wheat seedlings was evident from the fact that there is an overall decrease in germination percentage, root shoot length and its ratio, no of lateral rootlets produced, biochemical parameters. In all cases a negative correlation was obtained between concentration of the effluent and the biochemical parameters (Chlorophyll, Protein, Amino acid, Nucleic Acid and Carbohydrates).Same observation has also been reported by (Mahapatro and Mohanty, 2007; Indira and Mohanty, 2006) in green gram and ragi seedlings treated with ASCI, Aska effluents.

The present study also demonstrated that the efficient treatment of sugar mill effluent to Red gram and Wheat seedlings produced persistent morphological retardation. Moreover, decline in biochemical parameters attributed to the manifestations of decreases in biochemical parameters and decreases in root and shoot length in the seedlings of both crop plants.

Therefore, it is extremely necessary to treat the effluent properly before release to a river system for irrigation in agriculture to minimize harmful impact. (Sharma *et al.*, 2002; Rajesh, 1995; Pandey and Neraliya, 2002; Mishra and Pandey, 2002; Jothimani and Bhaskaran, 2002).

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