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Dual and Axenic culture of *Sclerospora graminicola* on Pearl Millet and *in vitro* raised disease resistant host germplasm

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ABSTRACT

Mother axis of the infected suppressed ear-head of pearl-millet with embryoids was sliced (2-3 mm) and surface sterilized with 0.2 % Ethyl mercuric chloride followed by repeated washings with sterile antioxidant solution (50 mg/L citric acid, 25 mg/L ascorbic acid and 50 mg/L P.V.P). Sterilized slices were cultured on modified MS medium supplemented with 10 mg/L IAA, 0.5 mg/L Kinetin, 2.5 mg/L 2,4-D, 3.0 mg/L casein hydrolysate, 25 mg /L ascorbic acid and 150 mg/L coconut water. Two types of calli were formed after 10-15 days. Slow growing compact milky coloured was embryogenic and the other hyaline fast-growing was non-embryogenic. The fungus grew on the callus after 20-25 days of inoculation. It grew axenically on the surface of the medium after sometime. Slow growing embryogenic callus was subcultured to auxin free ¾ strength MS medium with 25 mg/L ascorbic acid, 100 ml/L coconut water and 0.5 mg/L Kinetin. Regeneration plantlets were transferred for rooting on ½ strength MS medium containing 1.5 mg/L IBA and 25 mg/L ascorbic acid. The regenerated plantlets with strong root system were transferred to 4 earthen pots. The plantlets raised were subjected for disease resistance by growing them on the medium containing different concentrations of the fungus filtrate. The plantlets showing resistance have been accepted as diseases resistant.

INTRODUCTION

Green-ear and downy mildew disease caused by *Sclerospora graminicola* (Sacc.) Schroet is one of the most important diseases in bajra (Pearl-millet)growing tracts of India and cause considerable damage to this crop (Cummings, 1975). The object of the present investigation was to grow *Sclerospora graminicola* on callus tissue raised from the mother axis of the infected suppressed and excessively proliferated ear-heads of pearl millet and through dual culture of the host tissue and the fungus to obtainaxenic culture of the fungus. Attempt was made to enhance saprophytic growth of the fungus on more suitable growth medium and test the strain against the toxin released in the filtrate for growth of the regenerated host. Earlier limited saprophytic growth of the fungus was obtained which was not sufficient enough to carry out further studies specially to develop disease resistant germplasm of the host through its regeneration (Tiwari and Arya, 1969; Arya and Tiwari, 1969; Arya and Shekhawat, 1981). Vasil and Vasil (1982) isolated, cultured and regenerated the protoplast of *Pennisetum americanum* and characterized the somatic embryos into embryogenic and nonembryogenic calli. Earlier Rangan (1974, 1976) reported regeneration of tissue cultures of some Indian millets like *Panicum miliaceum*, *Eleusine coracana* and *Pennisetum typhoideum* on MS medium (Murashige and Skoog, 1962) supplemented with2, 4-D and coconut water. Related to regeneration an important information was available on diffusion of physiological gradients for clustering of cells leading to embryogenesis (Ross and Thorpe, 1973). Halparin (1970) showed that embryogenesis was induced in carrot callus growing in auxin containing medium in the suspension culture only. Nutrient media used for the initial proliferation of the tissue played a vital role in initiating embryogenesis. Suitable callus growth is obviously related to subsequent organogenesis of embryogenesis from diverse species of plants *in vitro* culture (Bhansal and Arya, 1979; Karnosky, 1981; Thorpe, 1985; Gupta and Darzan, 1987; Arya and Shekhawat, 1986; Arya and Shekhawat, 1987).

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Carlson (1980) was first to demonstrate that plant cells and protoplast could be selected to culture for use of resistant cell lines which may be regenerated with altered response to toxicity of the pathogen. Since then considerable interest has been generated in the potentiality of callus culture and its prospects for regeneration and development of diseases resistant germplasm. Results obtained are presented in the paper.

MATERIALS AND METHODS

The grains were collected from partially proliferated spikelets of pearl millet infected by *Sclerospora graminicola* along with infected tillered shoots which were excessively proliferated with severely arrested growth. This suppressed ear head formation was considered as the advanced stage of green ear disease. Explants of the size 2-3mm were cut from suppressed ear heads for raising dual cultures with embryoids. For raising dual cultures, infected grains were also used. These were surface sterilized with 0.2 % ethyl mercuric chloride, rinsed with sterile distilled water containing antioxidant solution (25 mg/L ascorbic acid; 50 mg/l citric acid; 50 mg/L (PVP) polyvinyl pyrrolidon). Sterilized explants were transferred to the MS medium containing 10 mg/L IAA, 0.5 mg/L kinetin, 2.5 mg/L 2,4-D, 3.0 mg/L casein hydrolysate, 25.0 mg/L ascorbic acid and 150 mg/L coconut water. The pH was adjusted to 5.8 before autoclaving to 15 lbs/sq. inch pressure for 20 min. The cultures were incubated in lighted culture chamber in continuous light of 3000 lux maintaining 26 ± 2 °C and 50-70% relative humidity.

DUAL CULTURE

Isolation and subculture of diseases host tissue

From seeds

Callus initation on the medium took place after 10 days of seed germination. Callus formed was distinguished into two types (i) slow growing with milky colour and compact texture and (ii) fast growing hyaline and fragile. Slow growing callus was separated from fast growing callus and subcultured in ¾ strength of the auxin free MS medium for regeneration.

Fungus from suppressed ear-heads

Callus appeared on the periphery of the explants after 2-3 weeks and grew slowly. Like previous case two types of calli appeared viz. (i) slow growing embryogenic which was subcultured to auxin free MS medium containing 0.5 mg/L kinetin + 5.0 mg/L casein hydrolysate + 15 % coconut water + 25 mg/L ascorbic acid. Fast growing non-embryogenic callus was discarded. Initation of the fungal growth on embryogenic callus was slow but after 40 to 60 days it grew vigorously (Fig. 1).

AXENIC CULTURE

The fungus growing on the callus surface of the medium was subcultured on the MS medium (liquid and solid) supplemented with 0.5 mg/L kinetin + 10.0 mg/L 2,4-D + 15 % coconut water + 25 mg/L ascorbic acid + 1.0 mg/L biotin + 3.0 mg/L casein hydrolysate + 150 mg/L coconut water. After 2-3 weeks of incubation at 24 ± 2 °C colonies of the fungus were formed which were cottony white (Fig. 2). The inoculated liquid culture was given mild shake on reciprocating shaker (5 g).

Microscopic study of the fungus revaled typical sporangiophores and sporangia of *S. graminicola* as observed earlier (Tiwari and Arya, 1969). The sexual structures viz. antheridia and oogonia were formed in abundance from coenocytic mycelium resulting in oospore development.

Three weeks old callus raised from healhy grains on the MS medium was inoculated with the fungus from axenic culture. The fungal infection was established within two weeks and the fungus turned saprophytic on the above given medium.

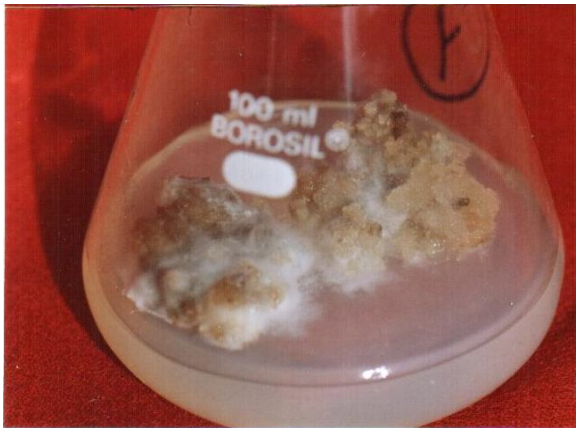

Fig. 1: Photograph showing Dual culture (fungus and host) with embryogenic callus culture.

Fig. 2: Photograph showing axenic culture of Sclerospora graminicola on liquid MS medium with casein hydrolysate (3g/L) and biotin (1.5 mg/L).

RESULTS

Callus induction

Callus initation was obtained after 6 days of inoculation of experimental material like grains but the explants from infected organs induced callus after 14 days of inoculation. Different combinations of auxins (2, 4-D and NAA) and cytokinins (Kn and BAP) which were used for initation of the callus are given below:

(a) MS + 2, 4-D (2.0 to 10.0 mg/L)

(b) MS + NAA (0.1, 1.0 to 6.0 mg/L)
(c) MS + Kn (0.05 to 2.0 mg/L)
(d) MS + BAP (0.05 to 2.0 mg/L)
(e) MS + 2, 4-D (1.5 to 3.5 mg/L) + Kn (0.05 to 1.0 mg/L)

The cultures were kept in the culture chamber at 26 ± 2 °C at 2000 lux light mixing fluorescent and incandescent light in the ratio of 4:1. Results obtained of callus initiation are presented in the tables 1 and 2. 2,4-D was found suitable for producing embryogenic callus. NAA produced slow growing callus in lesser amount as compared to 2, 4-D. Higher concentration of NAA showed inhibitory effect on callus development. Kn and BAP when incorporated separately in auxin free medium induced callusing. The callus produced, however was very small in quantity. The optimal was 0.5 mg/L with Kn and BAP. In the present studies 2, 4-D (2.5 mg/L) when combined with Kn (0.5 mg/L) gave best results. They yielded 980 mg in 3 weeks growth. Two types of calli were distinguished viz. embryogenic with slow growth and non-embryogenic with very fast rate of growth. Slow growing callus at the advanced stage developed embryoids after 6 days of growth and also turned light green in continuous light.

Callus morphogenesis

The embryoids obtained from healthy callus were transferred to ¾ strength MS medium without 2, 4-D supplemented with ascorbic acid (25 mg/L) and coconut water (150 ml/L). Different concentrations of Kn and BAP used for morphogenesis of callus are given in the following:

(a) ¾ MS + Kn (0.1 to 1.0 mg/L)
(b) ¾ MS + BAP (0.1 to 1.0 mg/L)
(c) ¾ MS + Kn (0.1 to 1.5 mg/L) + BAP (0.1 to 1.5 mg/L).

10 replicates were used for each treatment and tissues were grown for three successive passages at the same concentrations to remove the residual effect of pre treatment of culture and they were subcultured every 2 weeks on fresh medium.

The cultures were kept in the light chamber at 30 ± 2 °C and 3000 lux light mixing fluorescent and incandescent. On transfer of callus to ¾ MS medium supplemented with cytokinin (BAP and Kn), separately in different concentrations produced shoots (Table 2).

The Kn with 0.4 mg/L showed best results and 72 % of embryos produced shoots. BAP and Kn combined produced shoots but the results were poor (10-18%).

Root induction

Shoot segments were transferred to the fresh ½ MS medium for root induction with IAA or IBA incorporated in the medium. Rooting was initiated after incubation for 6-7 days at 28 ± 2 °C. Various combinations of auxins and cytokinins were used. Results obtained are presented in the table 3. IAA and IBA gave the positive response to rooting from the distal end of the shoot segments. IAA with optimal at 2.5 mg/L gave the best results. Cytokinins however showed no response for root induction.

***In vitro* hardening and acclimatization of tissue culture regenerated plants**

After root formation, the *in vitro* regenerated plantlets were ready for transfer from aseptic culture to the field. These were prepared for further growth, hardening and acclimatization and the establishment for autotrophic adaptation before transfer to the field. Rooted plantlets were removed after 2 weeks of culture from the rooting medium and transferred to the reduced salt concentration (1/4) of MS medium eliminating vitamins, carbon source i.e. sucrose and growth regulators. The tubes with plantlets were kept in glass house under bell jar covered with wet cloth to keep the outer atmosphere humid with 80-90% relative humidity. The temperature in the green house varied from 25 to 40 °C (night to day) and highest intensity from 0 to 4000 lux. The lower half of the test tube was covered with black carbon paper to provide complete darkness to facilitate root development and growth. After 10 days, the plantlets were carefully taken out from the medium without causing any damage to the delicate root system. They were then washed thoroughly under running tap water to remove all the adhering nutrient agar. The plantlets were treated with a systemic fungicide Bavistin (0.05 %) for 10-15 min and finally rinsed with distilled water and kept for 10-15 min transplanted to 6'' earthen pots containing vermiculite and sandy soil in the ratio of 3:2. The pH of the soil was maintained 4.8 to 6.0. The pots were watered with tap water regularly and once or twice with Hoagland solution. The results suggested that this method might be developed for *in vitro* propagation of *P. americanum* which might be exploited for genetic manipulation of the species.

Development of disease resistant cell lines

A-Embryoids formed in embryogenic dual culture were isolated and grown on modified MS medium with different concentration of $MnCl_2$ (50-400 mg/L) with Kn (0.5 mg/L), ascorbic acid (25 mg/L), coconut water (150 mg/L) and a fungicide Bavistin (0.05 %) for further morphogenesis of embryoids. The cultures were kept in culture chamber at 30 ± 2 °C and 3000 lux light intensity. Six replicates were used for each treatment and tissues were grown for 3-4 successive passages to remove the residual effect of pretreatment of auxins.

The embryoids turned green and regenerated and formed shoots in continuous light a mixture of incandescent and fluorescent light in the ratio of 1:4. The results obtained are presented in the table-4 and fig.3. The results showed that $MnCl_2$ (200 and 250 mg/L) + Bavistin (0.05 %) were found best for controlling fungus growth. Bavistin prevented growth of the fungus and $MnCl_2$ changed the physiology of diseased tissue to normalcy. The growing shoot segments (3-5cm) were transferred to fresh ½ MS medium for root induction with IBA (1.5 mg/L) ascorbic acid and $MnCl_2$ (250 mg/L). The rooting was initiated to form plantlets after incubation for 6-7 days at 28 ± 2 °C (Fig. 4). The plantlets with developed root system are transplanted to 6'' earthen pots. The process of root hardening, acclimatization and transplantation has been discussed earlier.

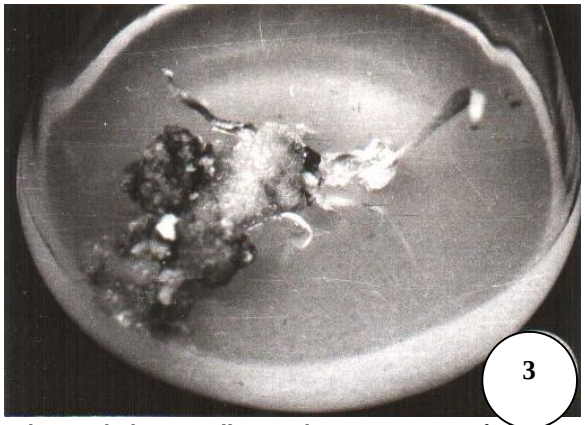

Fig. 3: Photograph showing callus morphogenesis on auxin free MS medium with Kinetin, ascorbic acid and coconut water.

B- Embryoids formed in embryogenic normal culture were picked up and transferred to MS medium containing Kn (0.5 mg/L) + ascorbic acid (25 mg/L) and 150 mg/L coconut water containing filtrate of cultures from saprophytic growth of the fungus *Sclerospora graminicola.* Filtrate from the fungus culture was extracted, through sterile muslin cloth and centrifuged for 15 min at 4°C. The sterile filtrate was used for experiments. Various concentrations of aseptic filtrate (20-300 mg/L) were incorporated into above mentioned callus differentiating medium. The cultures were kept in light culture chamber at 30 ± 2 °C and 3000 lux light intensity. The embryoids were regenerated and formed shoots in continuous light. The cultures with 150 ml/L filtrate showed the critical stage of regeneration at which only 5% embryoids showed regeneration and 95% succumbed to toxic effect of the fungus filtrate. Cultures with above 200 ml/l concentration of the filtrate proved toxic to the embryoids and did not regenerate to form shoots (Table-5).

The regenerated plants (1 month old) were transplanted to 6'' earthen pots with oospore mixed soil. The pots were kept covered with polyethylene bag and watered with distilled water for the first one week then with tap water. Grains were collected after maturation from healthy plants and sown for further testing.

(b) Grains collected from above experiments were taken and after surface disinfection were sown on the ¼ strength MS medium. The plantlets were transferred to the above medium supplemented with 150 mg/l filtrate of the fungus and surviving plantlets were grown for 2 weeks. Onwards plantlets were taken out washed with distilled water and transferred to soil for growth. Grains formed were collected.

It was observed that 90-95 % plants upto F_2 generation showed their resistance to downy mildew disease. The experimental results need further confirmation and detailed studies.

Table. 1: Combined effect of 2,4-D and Kn of callus induction of Pearl Millet.

Conc. of 2, 4-D	Kinetin (mg/L)							
	0.05		0.1		0.5		1.0	
	Nature of Response	% of Response	Nature of Response	% of Response	Nature of Response	% of Response	Nature of Response	% of Response
1.5	C+	8	C+	10	C++	22	C+	11
2.0	C+	11	C++	12	C+++	41	C++	28
2.5	C++	16	C++	18	C++++	57	C++	12
3.0	C+	6	C++	22	C+++	32	C++	18
3.5	C+	7	C+	11	C++	28	NR	-

NR= No response, C = Callusing, += Poor callusing, ++= Moderate callusing, +++= Good callusing, ++++ = Excellent callusing

Table. 2: Effect of cytokinins on callus morphogenesis of pearl millet.

S. No.	Concentration used (mg/L)	Cytokinin used			
		Kinetin		BAP	
		Nature of response	% of response	Nature of response	% of response
1.	0.1	S+	2	NR	-
2.	0.2	S++	16	S	8
3.	0.3	S+++	28	S	12
4.	0.4	S++++	72	S	22
5.	0.5	S++++	42	S	11
6.	0.6	S+++	31	S	29
7.	0.7	S++	21	S	42
8.	0.8	NR	-	S	7
9.	0.9	NR	-	NR	-
10.	1.0	NR	-	NR	-

NR= No response, S= Shoot, += Poor shooting, ++ = Moderate shooting, +++ = Good shooting, ++++ = Excellent shooting

Table. 3: Effect of auxins on root induction of Pearl Millet.

Concentration used (mg/L)	Auxins used			
	IAA		IBA	
	Nature of response	% of response	Nature of response	% of response
0.5	NR	-	NR	-
1.0	R+	9	R	7
1.5	R+	8	R	18
2.0	R+++	38	R	25
2.5	R++++	56	R	16
3.0	R++	28	NR	-
3.5	NR	-	NR	-

NR= No response, R= Root, += Poor rooting, ++= Moderate rooting, +++= Good rooting, ++++= Excellent rooting

Table. 4: Effect of $MnCl_2$ on shoot formation from embryoids from embryogenic dual culture.

$MnCl_2$ Concentration (mg/L) with 0.05 % Bavistin	*No. of shoots formed
50.00	12
100.00	16
150.00	32
200.00	62
250.00	66
300.00	36
350.00	20
400.00	08

*Average number of 6 replicates

Table. 5: Effect of fungus culture filtrate on shoot formation from embryoids in embryogenic normal culture.

Fungus filtrate concentration (ml/L)	*percent shoots formed
20.00	40.0
50.00	40.0
75.00	30.0
100.00	20.0
150.00	05.0
200.00	00.0
250.00	00.0
300.00	00.0

*Average number of 6 replicates.

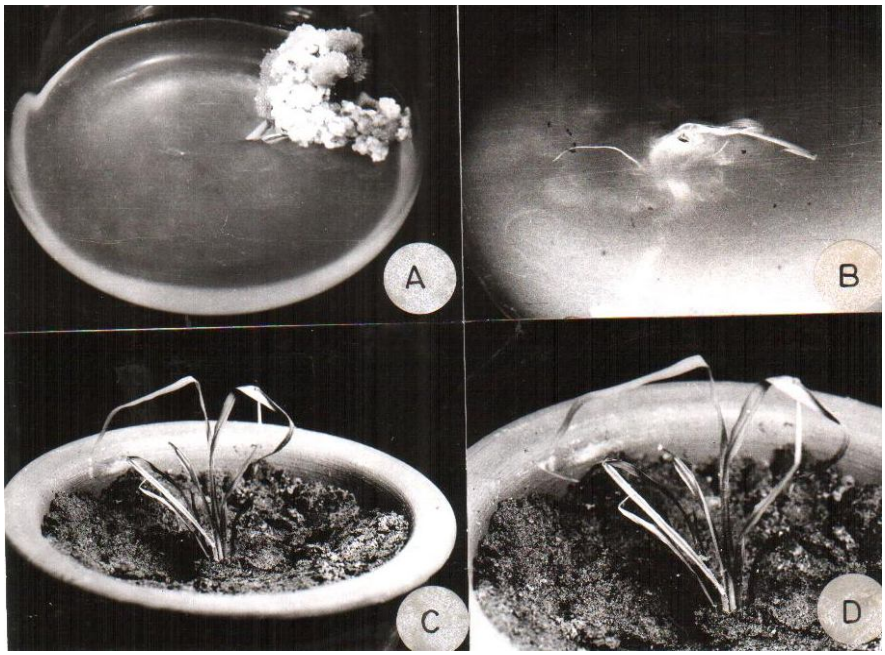

Fig. 4: Photograph showing root initiation on MS medium supplemented with IBA (1.0 mg/L). (A-B: Root initiation, C-D: Plantlet transferred to pots containing sandy soil and grown under controlled conditions.

DISCUSSION

Results presented in the paper need discussion on two important points viz. dual and axenic culture of the fungus and *in vitro* callus establishment of the host, its regeneration and development of disease resistant host cell lines.

During experimentation dual culture of the host and pathogen was obtained by growing the infected host tissue from suppressed ear-head diseased grains on modified MS medium supplemented with 2, 4-D, Kn and ascorbic acid. For axenic culture of the fungus additionally casein hydrolysate coconut water and biotin were added to the above medium. This enabled the authors to obtain antotrophic growth of the fungus like any other saprophyte. Limited growth of *Sclerospora graminicola* on dual and axenic culture has been reported earlier by our group (Tiwari and Arya, 1969; Arya and Tiwari, 1969; Arya and Shekhawat, 1981). Similar observations were made in *Sclerospora sacchari* (Leu, 1978; Chen et al., 1929), *Perenospora farinose* (Ingram and Joachim, 1971). *In vitro* regeneration of plants particularly monocotyledons has been rather recalcitrant. Vasil and Vasil (1982) were the first to characterize the embryogenic cell culture derived from cultured inflorescence of *Pennisetum americanum*. Following the technique the callus was raised out of cultivar BJ-104 being highly susceptible to downy mildew disease. The tissue as stated in the text were identified into two groups:

1. Fast growing non-embryogenic and

2. Slow growing embryogenic calli. The slow growing embryogenic callus with dual culture i.e. containing fungus was used for testing its resistance. Following two methods were used as given in the text.

a. Embryoids developed in the embryogenic dual cultures were isolated and grown on modified MS medium with supplements like Kn, ascorbic acid, coconut water along with $MnCl_2$ (250 mg/l) and Bavistin (0.05 %) in the medium. The embryos were regenerated into plantlets which were grown to form inflorescence resulting in healthy grain formation.

b. The cells from the embryogenic callus were grown on the regenerating modified MS medium supplemented with 150 ml/L of the fungus filtrate into the culture medium which supported saprophytic growth of *Sclerospora graminicola.* Regenerating cell groupswere picked up for plantlet formation. The plantlets were allowed to grow and flower. The disease resistant strains were picked up for raising resistant cultivar lines. The experimental results need further confirmation and experimentation.

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