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Effect of Media, pH, Temperature and Light on the Growth of *Coniothyrium minitans* (Campbell 1947) – A Novel Biocontrol Agent for Cabbage Head Rot caused by [*Sclerotinia sclerotiorum* (Lib.) De Bary]

K. Sivagnanapazham[1*], M. Karthikeyan[1], T. Raguchander[1], 3 R. Swarnapriya[2] and A. Kamalakannan[1]

[1]Department of Plant Pathology, [2]Department of Vegetable Science, TNAU, Coimbatore, India

**Corresponding author*

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A B S T R A C T

Cabbage head rot (White mould) disease caused by *Sclerotina sclerotiorum* is a potential threat to cabbage production and 60 percent yield loss was recorded. The disease was first reported in Kodaikanal area in Tamil Nadu. *Coniothyrium minitans* is an novel biocontrol agent for the management of *Sclerotinia sclerotiorum* because of its sclerotial mycoparasites. The growth of *Coniothyrium minitans* was studied with the different growth parameters like media, pH, temperature and light. The results revealed that the fastest mycelial growth was observed in Potato Dextrose Agar Media (26.33). Among the different media tested, the sporulation was observed in the PDA medium at the pH level of 5.5, temperature 20^{o}C with the 12hrs dark and light condition.

Introduction

Cabbage is one of the most widely cultivated temperate vegetable crop in India. India is the second largest producer of cabbage in the world next to china. The major Cabbage producing states in India which includes West Bengal, Tamil Nadu, Odisha, Madhya Pradesh, Bihar and Assam (NHB- APEDA 2018). In terms of area and production West Bengal ranks first with an account of (79.46000Ha/ 2288.50MT) and in productivity Tamil Nadu ranks first with (66.07MT/Ha) followed by Uttar Pradesh, Himachal Pradesh, Jammu and Kashmir, and West Bengal (Horticultural Statistics Division, DAC&FW). In Tamil Nadu, the crop was cultivated in The Nilgiris, Coimbatore, Krishnagiri, Theni, Erode and Dindigul Districts with a total area and production of 1400 Ha and 78,600 tonnes respectively(www.data.gov.in). The crop is subjected to many foliar and soil borne diseases, among the soil-borne fungal

diseases, Cabbage head rot (White mould) disease caused by *Sclerotinia sclerotiorum* is a potential threat to cabbage production and limits the crop cultivation. Yield loss recorded upto 60 percent. The disease occurrence was first reported in Kodaikanal area of Dindigul district in Tamil Nadu (Alagianagalingam *et al.,* 1978). It causes severe infection in both fields as well as storage conditions.

Coniothyrium minitans was first described by Campbell in 1947 in California as a biological control of *Sclerotina sclerotiorum* and it was isolated from sclerotia present in the soil. Ecologically it is a fastidious mycoparasite (Whipps *et al.,* 2008) on the sclerotia of many ascomycetous fungi such as *Sclerotinia sclerotiorum, Sclerotinia minor, Sclerotinia trifoliorum, Sclerotinia cepivorum, Botrytis sp.* and not able to parasitise the sclerotia of Basidiomycetes fungi (Whipps and Gerlagh 1992). *C. minitans* has a potential to degrade the sclerotia in the soil and it reduces the viability and decreasing the carpogenic germination of *S. sclerotiorum* (Whipps and Budge 1990; Jones and Whipps 2002). This mycoparasite is promising biocontrol agent for *S. sclerotiorum*, and has been shown to control the pathogen at low disease levels in both glasshouse and field trials (Huang, 1980; Whipps, Budge and Ebben, 1989; Budge and Whipps, 1991; Whipps and Budge, 1992; Budge *et al.,* 1995; Evenhuis *et al.,* 1995; McQuilken and Whipps, 1995; McQuilken *et al.,* 1995).

Information on the studies pertaining to the effect of environmental factors for conidial germination, pycnidial production and hyphal extension was limited (Whipps and Gerlagh, 1992). Since, the environmental factors play a major role to restrict the activity of biocontrol agents. Therefore, the environmental factors are essential to improve the efficacy and large scale inoculum production of *C. minitans*.

This paper describes the different culture media and environmental factors such as different temperature, pH and light on conidial germination, pycnidial production and hyphal extension of *C. minitans.*

Materials and Methods

Survey

A rowing survey was conducted during 2018 - 2019 in order to understand the magnitude of the occurrence of head rot symptoms in cabbage growing districts of Tamil Nadu. Disease incidence was recorded in The Nil Gris, Coimbatore and Kodaikanal and the infected samples and sclerotia was collected to isolate the biocontrol agent (Table 1).

Isolation of *Coniothyrium minitans*

Baiting technique was used to isolate the *C. minitans* from sclerotia of *S. sclerotiorum*. The sclerotia was collected from the white mould infected cabbage and surface sterilized with 1% sodium hypochlorite and it was placed in sterilized autoclaved sand containing petriplate.

Then the sclerotia was sprayed with spore suspensions of *S. sclerotiorum* and incubated for 40-45 days. In order to maintain the adequate humidity 8ml sterile Distilled water was sprayed at weekly intervals (Sandys-Winsch *et al.,* 1993).

Preparation of Spore suspensions

The spore suspension of *S. sclerotiorum* was prepared by scrapping gently the petri dish containing culture along with sterile distilled water.

The concentration of spore suspension $1x10^{-6}$ ml was counted by using Haemocytometer (Yang *et al.,* 2007).

Effect of media on the growth of *Coniothyrium minitans*

The growth of *C. minitans* was examined from its conidial germination, pycnidial production and hyphal extension at room temperature (28 ± 2^{o}C) by using different media like PDA (Potato Dextrose Agar), CZA (Czapek Dox Agar), H.CZA (Half Strength Czapek Dox Agar), OMA (Oat Meal Agar), MEA (Malt Extract Agar), WA (Water Agar), V8 Juice Agar and CDA (Carrot Dextrose Agar) with three replications. The observations were made with ten days intervals (Whipps and Gerlagh, 1992).

Effect of pH on the growth of *Coniothyrium minitans*

PDA media was prepared and the media pH was adjusted into different pH levels viz., 4.0,4.5, 5.0, 5.5, 6.0, 6.5 with 0.1N Hcl or 0.1 N NaOH and autoclaved at 121.5 ^{o}C for 20 mins with 15 psi. 20 ml of media with different pH level was poured into sterilized petri dish and allowed to solidify. 9mm disc of *Coniothyrium* culture was placed at the centre of the petridish by using cork borer and the plates were incubated at 20^{0}C and room temperature 28±2^{o}C.Three replications were maintained and radial growth of mycelium were recorded at 10 days interval (Mark P. Mcquilken *et al.*, 1997)

Effect of Temperature and Light on the growth of *Coniothyrium minitans*

Fifteen days old culture of *C. minitans* was inoculated into PDA media containing petri plate and incubated at two different temperatures 20^{0}C and room temperature 28±2^{o}C. The radial mycelial growth and number of pycnidia produced per plate were recorded. Each treatment was made with three replications. To assess the growth of *C. minitans* in light the plates were incubated with alternate day and light with 12 hrs darkness.

Assessment of Pycnidial production and Hyphal extension of *Coniothyrium minitans*

The mycelial growth and pycnidial production of *C. minitans* was examined by using 8 different media PDA (Potato Dextrose Agar), CZA(Czapek Dox Agar), H.CZA(Half Strength Czapek Dox Agar), OMA(Oat Meal Agar), MEA(Malt Extract Agar), WA(Water Agar), V8 Juice Agar and CDA(Carrot Dextrose Agar). 9 mm disc of *C. minitans* was placed the petri plate containing 8 different media and incubated at 20^{0}C and room temperature 28±2^{o}C. The observations were made at 10 days interval.

Statistical analysis

All the data were analyzed using of variance and treatments means compared with Duncan's Multiple Range test at a probability of 5%.

Results and Discussion

Isolation of *Coniothyrium minitans*

The sclerotia of three isolates from ketti, Palada and Thadiyankudisai collected during survey were able to produce *C. minitans.* Among these the Thadiyankudisai isolates were used for further studies (Table 2: Plate 1).

Effect of medium

The mycelia growth was tested with different media at room temperature (28+2^{o}C). The results revealed that the maximum mycelia growth of *C. minitans* was observed at PDA (26.33) followed by V8 juice agar (24.33), CDA (24.00), OMA (19.95), MEA (19.16) and H.CZA (18.48). The lowest mycelia

growth 17.30 and 10.68 was observed in CZA and WA respectively

The Maximum Hyphal extension and growth was observed in Potato dextrose agar (28.67) followed by Half Strength Czapek Dox Agar (28.33) and pycnidial production was quickly observed in Potato Dextrose Agar (PDA) (2 Nos of pycndia) (Table 3, Plate 2)

Effect of pH

Since the maximum mycelial growth of *C. minitans* was observed in PDA media, the pH of the media with different level 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 was also performed. The results revealed that the maximum mycelia growth was occurred in media pH 5.5 (34mm) followed by pH 6.5 (33mm), pH 6.0 (32mm), pH 5.0 (27mm), pH 4.5 (23mm). The minimum growth was observed in pH 4.0 (14mm) (Table 4, Plate 3)

Effect of temperature and light

The growth of mycelia was assessed in two different temperatures, room temperature 28±2^{o}C and 20°C. The results revealed that the growth of *C. minitans* was very well grown in 20°C when compared with room temperature. The effect of temperature shows that hyphal extension and pycnidial production was maximum in 20°C when compared with room temperature. By increasing light period from continuous dark to light (12hrs dark / 12 hrs light) there is a formation of pycnidial productions was observed (Table 5, Plate 4&5).

Table.1 Survey and collection of *Sclerotinia sclerotiarum* (Head rot) isolates from cabbage growing area in Tamil Nadu, India

S.No	Location	District	Latitude	Longitude	Isolate description	Percent disease incidence
1.	Narasipuram	Coimbatore	10.9942	76.7996	CBESS1	00.0^{i} (1.62)
2.	Ketti	The Nilgris	11.3680	76.7285	KETSS2	23.4^{g} (28.93)
3.	Muthorai	The Nilgris	11.4557	76.6402	MTRSS3	28.3^{f} (32.14)
4.	Palada	The Nilgris	11.3680	76.7285	PALSS4	17.8^{h} (24.96)
5.	Nanjanadu	The Nilgris	11.3673	76.6408	NAJSS5	**72.1^{a} (58.12)**
6.	Kappathorai	The Nilgris	11.3673	76.6572	KAPSS6	34.2^{d} (35.79)
7.	Emerald	The Nilgris	11.4916	76.7336	EMRSS7	**38.6^{c} (38.41)**
8.	Poombarai	Dindigul	10.2564	77.4078	PMBSS8	**41.8^{b} (40.28)**
9.	Vilpatti	Dindigul	10.2694	77.4977	VILSS9	31.2^{e} (33.96)
10	Thadiyankudisai	Dindigul	10.3632	77.9824	TDKSS10	22.3^{g} (28.18)
SED						0.40
CD						0.85

Table.2 *Coniothyrium minitans* producing isolates from sclerotia of *Sclerotinia sclerotiorum*

S.No	Location	District	Isolate description	Presence of *Coniothyrium minitans*
1.	Narasipuram	Coimbatore	CBESS1	-
2.	Ketti	The Nilgris	KETSS2	+
3.	Muthorai	The Nilgris	MTRSS3	-
4.	Palada	The Nilgris	PALSS4	+
5.	Nanjanadu	The Nilgris	NAJSS5	-
6.	Kappathorai	The Nilgris	KAPSS6	-
7.	Emerald	The Nilgris	EMRSS7	-
8.	Vilpatti	Dindigul	PMBSS8	-
9.	Poombarai	Dindigul	VILSS9	-
10.	Thadiyankudisai	Dindigul	TDKSS10	+

Table.3 Mycelial growth of *Coniothyrium minitans* (TDKSS10) isolate in different media at room temperature (28+2^{o}C)

S.No	Different media	10thday (mm)*	20thday (mm)*	30thday (mm)*	40thday (mm)*	50thday (mm)*	60thday (mm)*	Mean (mm)*
1	PDA	15^{b}	25^{a}	27^{a}	28^{a}	30^{a}	33^{b}	**26.33**
2	MEA	10^{d}	12^{f}	16^{g}	20^{c}	25^{c}	32^{b}	**19.16**
3	CZA	0.8^{e}	15^{e}	19^{e}	21^{c}	23^{d}	25^{e}	**17.30**
4	H.CZA	0.9^{e}	19^{d}	20^{d}	20^{c}	23^{d}	28^{d}	**18.48**
5	WA	0.2^{f}	0.9^{g}	11^{h}	13^{d}	18^{e}	21^{f}	**10.68**
6	OMA	0.7^{e}	12^{f}	17^{f}	25^{b}	30^{a}	35^{a}	**19.95**
7	CDA	12^{c}	23^{b}	25^{b}	27^{a}	28^{b}	29^{cd}	**24.00**
8	V8	20^{a}	22^{c}	23^{c}	24^{b}	27^{b}	30^{c}	**24.33**
	SED	0.18	0.49	0.38	0.53	0.54	0.69	
	CD	0.38	1.05	0.83	1.13	1.17	1.49	

Values are the means of three replications. **Values in the parenthesis are arc sign transformed values. In the column, Means followed by a common letter are not significantly different at 5% level by DMRT

Table.4 Mycelial growth of *Coniothyrium minitans* isolate in different pH level on PDA medium

S.No	pH	Mean(mm)*
1.	4.0	14^{e}
2.	4.5	23^{d}
3.	5.0	27^{c}
4.	5.5	34^{a}
5.	6.0	32^{b}
6.	6.5	33^{ab}
	SED	0.48
	CD	1.08

Table.5 Mycelial growth of *Coniothyrium minitans* (TDKSS10) isolate in different media at 20°C

S.No	Different media	10th day (mm)*	20th day (mm)*	30th day (mm)*	40th day (mm)*	50th day (mm)*	60th day (mm)*	Mean (mm)*
1	PDA	18^{b}	25^{a}	30^{a}	32^{a}	33^{ab}	34^{c}	**28.67**
2	MEA	12^{d}	17^{e}	23^{d}	27^{b}	32^{b}	38^{ab}	**24.83**
3	CZA	1^{f}	15^{f}	18^{f}	20^{d}	25^{d}	30^{d}	**18.17**
4	H.CZA	15^{c}	24^{b}	30^{a}	32^{a}	33^{ab}	36^{bc}	**28.33**
5	WA	11^{e}	19^{d}	28^{b}	33^{a}	34^{a}	37^{ab}	**26.67**
6	OMA	12^{d}	15^{f}	20^{e}	25^{c}	34^{a}	39^{a}	**24.17**
7	CDA	0.8^{f}	0.9^{g}	10^{g}	12^{e}	12^{e}	15^{e}	**8.45**
8	V8	20^{a}	23^{c}	25^{c}	27^{b}	29^{c}	30^{d}	**25.67**
	SED	0.69	0.43	0.52	0.66	0.52	1.01	
	CD	1.48	0.92	1.12	1.41	1.11	2.07	

Values are the means of three replications. **Values in the parenthesis are arc sign transformed values. In the column, Means followed by a common letter are not significantly different at 5% level by DMRT

Plate.1 Isolation and Growth of *C.minitans* in PDA medium

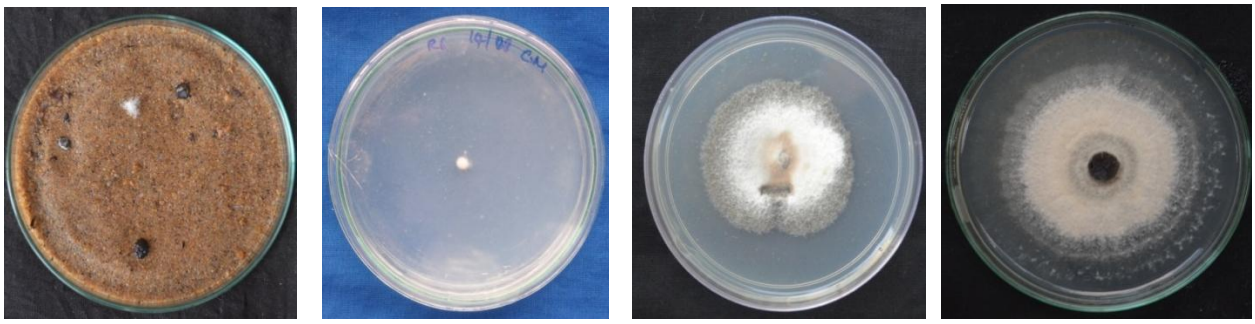


Plate.2 Growth of *C.minitans* in different media at room temperature (28± 2°C)

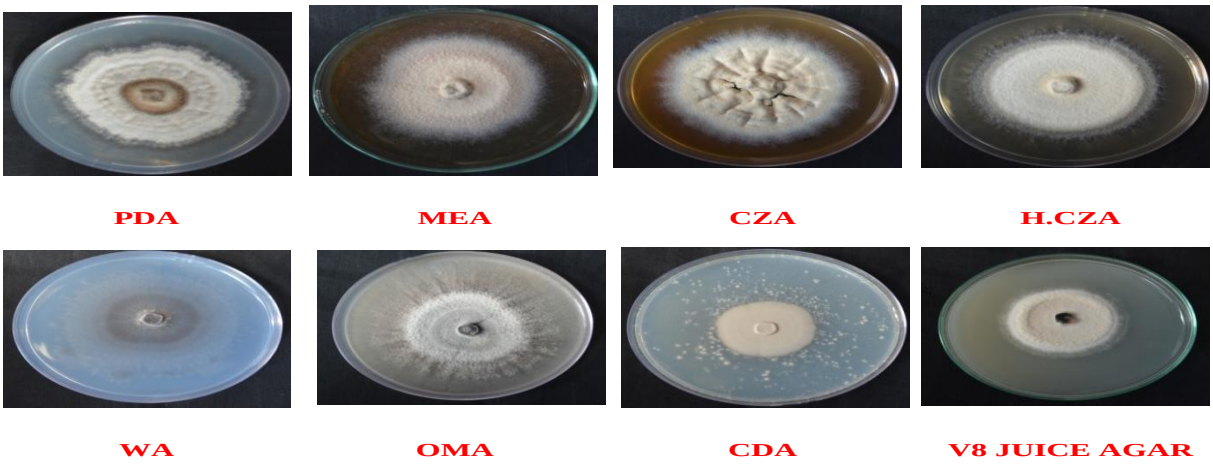


Plate.3 Growth of *C.minitans* at different pH in PDA medium

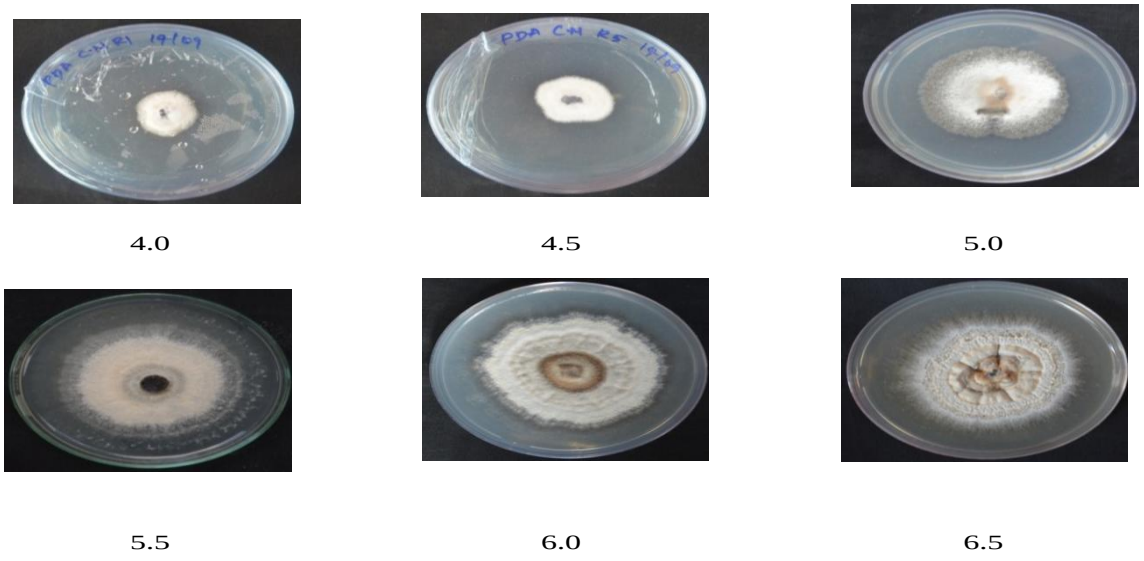


Plate.4 Growth of *C.minitans* in different media at 20^{o}C

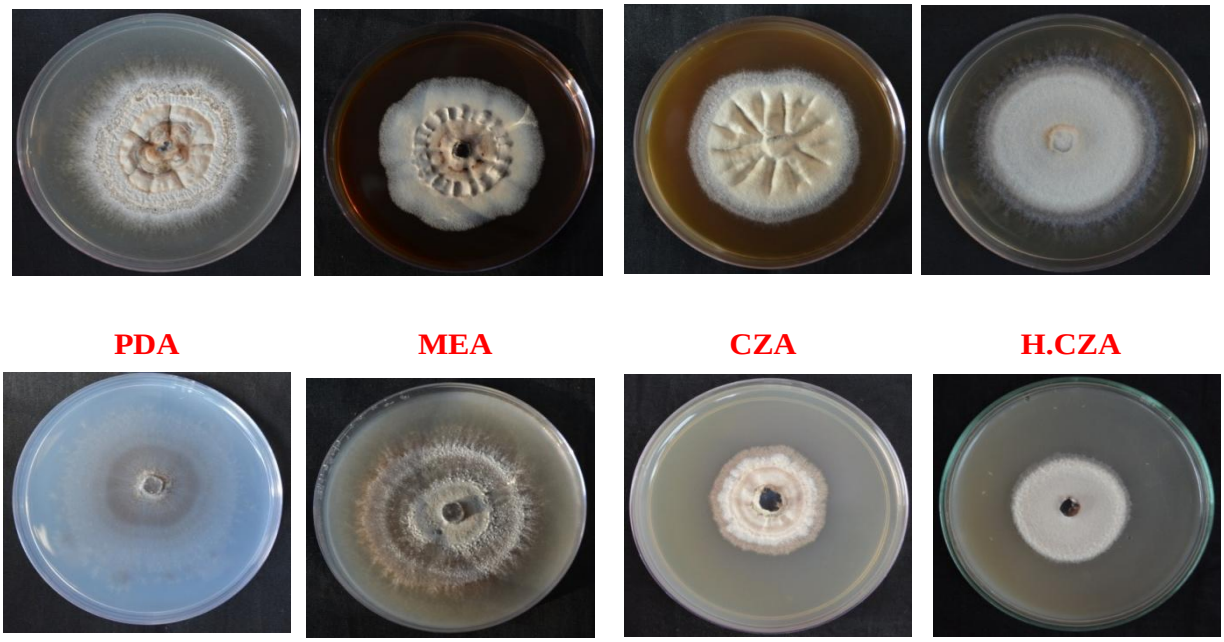
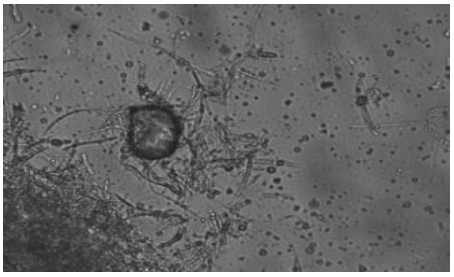
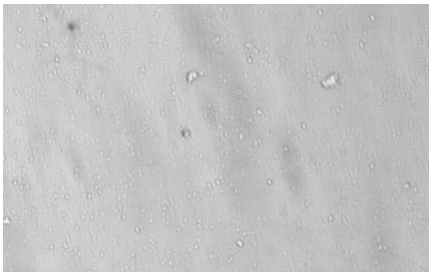


Plate.5 Pycnidia and pycnidiospores of *Coniothyrium minitans*

Potato dextrose agar medium

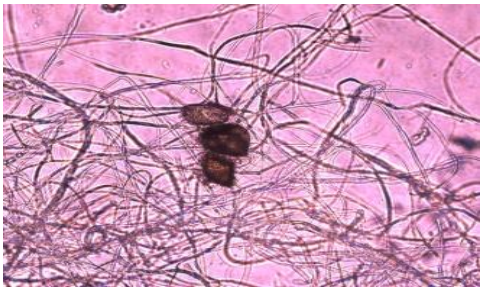


Pycnidia



Pycnidiospores

Half Strength Czapek Dox agar



Pycnidia

Pycnidiospores

The changes in the hyphal extension, conidial germination and pycnidial production were used to access the effect of growth media, pH, temperature and light of *C. minitans*. The simultaneous studies on the both growth and reproduction structures in culture may be the key factors for the commercial inoculum production. Though the Thadiyankudisai isolate was able to grow all the media tested, but the PDA is the suitable for conidial germination, Hyphal extension as well as pycnidial production, when compare to all other media tested. The same results were obtained by Mark.P. Mcquilken *et al* 1997 and Whipps and Gerlagh 1992. This confirm the suitability of this media for routine culture of this fungus and also suggest that they may be good media on which to base a more detailed study of the liquid culture of C. *minitans* Significantly the use of Molasses Yeast Broth for liquid culture of *C. minitans* has been reported by Papavizas, 1984 which interfere to PDA in terms of hyphal extension. As molasses yeast based liquid media are inexpensive relative to many others and also used for production of other biocontrol agents such as *Trichoderma viride* and *Pythium oligandrum*.

Since the maximum mycelial growth of C. *minitans* was observed in PDA media, the effect of pH of the media with different level 4.0, 5.0, 5.5, 6.0 and 6.5 results revealed that the maximum mycelial growth occurred in pH 5.5 followed by 6.5, 6.0, 5.0, 4.5. The minimum

growth was observed in pH 4.0. The results were correlates with the studies carried out by Mark P. Mcquilken *et al.,* 1997 and concluded that the *C. minitans* germinated between pH level 3.3 and 8.2 but the maximum germination occurred between pH 4.5 and 6.2 and also reported that the pycnidial production occurred between 3.3 and 8.2. This reports clearly indicated pycnidial production and hyphal extension occurred over a wide pH range 4 to 6 used for further inoculums production.

The growth of mycelia, hyphal extension and pycnidial production with two different temperatures room temperature (28+2^{o}C) and 20°C. The results revealed that the growth of *C. minitans* was very well grown in 20°C when compared with room temperature. The effect of temperature shows that hyphal extension and pycnidial production was maximum in 20°C when compared with room temperature. The results were compared with the studies carried out by Mark P. Mcquilken *et al* 1997. The temperature range for mycelial extension between 4°C and 25°C with maximum at 20°C-25°C. The temperature range for conidial germination and pycnidial production was 10°C-25°C narrower and 20°C is optimum. This temperature range for conidial germination on PDA is smaller than the range found for single isolate in Australia (Trutmann *et al* 1980).

Increasing the light periods has no effect on conidial germination or hyphal extension but significantly increases pycnidial production. Light has been reported to affect colony morphology and sporulation of *C. minitans* (Phillips, 1985). Increasing the period or intensity of light may well be a way to improve conidial production by *C. minitans* in the future.

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