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EFFECT OF VARIOUS CARBON AND NITROGEN SOURCES ON MYCELIAL GROWTH OF *Penicillium digitatum* ISOLATED FROM SWEET ORANGE GROWING AREAS OF TAMIL NADU

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ABSTRACT

Laboratory experiments were carried out at Deptt. of Plant Pathology, AC&RI, Killikulum, T.N.A.U. during 2015-16 to study the effects of various carbon and nitrogen sources on mycelial growth of *Penicillium digitatum* isolated from the green mould infected sweet orange fruits collected from Tirunelveli and Tuticori districts of Tamil Nadu to understand nutritional requirements and ecological survival of the isolates. Cultural characteristics of isolate 3 (as it was highly virulent) of *Penicillium* sp. were studied on eight different carbon and five different nitrogen sources (CYA solid and CYA liquid media). Three replications were maintained for each isolate at each treatment. Significant variation in the growth of *Penicillium digitatum* was recorded for both the carbon and nitrogen sources tested in solid and liquid form. Different carbon sources tried in this study revealed that sucrose (control) in CYA medium was the best carbon source which resulted in maximum mycelial growth of 90 mm in solid form for all the three isolates and in liquid form 0.98 g mycelial dry weight in isolate 3, 0.97 g in isolate 1 and 0.95 g in isolate 5. Different nitrogen sources used in this study indicated that sodium nitrate (control) in CYA medium was the best by recording significantly the maximum mycelial growth of 90 mm in isolate 3, 89.33 in isolate 1 and 90.00 in isolate 5 when used in solid form, while in liquid form maximum mycelial dry weight of 1.17 g in isolate 3, 1.06 g in isolate 1 and 1.03 g in isolate 5. It is summarized from this study that the sucrose as carbon source and sodium nitrate as nitrogen source is suitable for maximum growth of *Penicillium digitatum* which will be helpful in laboratory evaluation.

(Key words: Sweet orange, *Penicillium digitatum,* carbon source, nitrogen source)

INTRODUCTION

Penicillium digitatum is the most devastating pathogen of citrus fruit, being responsible for about 90% of production losses during post harvest handling according to the studies carried out by Macarisin *et al.*(2007). Agrios (2005), Ashok *et al.* (2007), Mekbib *et al.* (2007) also have reported significant losses during post harvest handling of perishables at varying level. In spite of the application of fungicides and the increased implementation of new biological control strategies, green mould continues to exhibit high infection pressure on stored citrus commodities worldwide.

Green mould of citrus, caused by *Penicillium digitatum* (Pers.:) Fr., Sacc, is one of the most economically important post harvest diseases of citrus worldwide (Palou *et al*., 2002). It is responsible for significant economic loss of fruits in the world (Bancroft *et al*., 1984). The pathogen may attack the fruit on the tree, in the packing house, in transit, in storage and in market (Plaza *et al*., 2003). Powell (1908) reported that the primary infection sites of *P. digitatum* are wounds inflicted on fruits during harvest and subsequent handling.

Penicillium enters tissues through wounds, but it can also spread from infected fruit by contact with healthy fruit through the uninjured skin (Agrios, 2005). Under humid conditions, the initial symptoms of *Penicillium* rots is the appearance of a soft, watery, slightly discoloured spot of varying size (approximately 0.5 cm-1.5 cm in diameter) on any part of the fruit (Olsen *et al.*, 2000). The discoloured spots enlarge from 2.5 cm to 5 cm in diameter after 1 to 2 days at 25 °C (Olsen *et al.*, 2000). Soon after, white mycelia appear on the surface of the fruit, near the centre of the spot and start producing blue or olive green spores. Soon, the entire fruit surface is rapidly covered with the spores, which are easily spread if the fruit is handled or exposed to air currents (Olsen *et al.,* 2000).

A single infected orange can be the source of infection to other oranges during storage and on transit (Jay, 2003). Common air moulds such as *Penicillium* species may gain entry into the susceptible tissue and cause loss during packaging (Ronald, 1988). Green mould caused by the pathogen *Penicillium digitatum* (Pers: Fr.) Sacc., is the most economically important postharvest disease of citrus. Actual losses due to green mould depend upon the area of production, citrus cultivar, weather and orchard conditions, and especially the extent of physical or mechanical injury to

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the fruit during harvest and subsequent handling. The conidia situated in injuries that ruptures oil glands or penetrate into the albedo of the peel usually bring irreversible infection within 48 h at 20-25^{0}C. Disease development was mediated by complex interactions between pathogen virulence mechanisms and host defense responses.

A high degree of variability in physiology and morphological characteristic enable *Penicillium digitatum* to occupy diverse ecological niches in many geographic regions. The earlier studies on green mould caused by *Penicillium digitatum* involved only reports of field observations on disease occurrence and its symptoms. Few workers have cultured a pathogen in laboratory and observed the colony morphology. Keeping these aspects in minds present work depicts the study of the effects of different carbon and nitrogen sources on mycelial growth of *Penicillium digitatum* isolated from the green mould disease affected fruits collected from Tirunelveli and Tuticori districts of Tamil Nadu to understand nutritional requirements and ecological survival of the isolates which will be helpful in laboratory evaluation.

MATERIALS AND METHODS

Isolation and characterization of *Penicillium digitatum*

The infected fruits were collected and used for isolation of pathogen by the method described by Zahara (2014). The fungus was purified by single spore isolation technique (Ho and Ko, 1997) and the purified isolates were maintained on PDA slants for further studies. The pathogen that appeared first was primarily identified using cultural and morphological features under the light microscope. The pathogenicity of isolated organisms was tested using matured fruits as per the methods described by Sharma *et al.* (1985). The six various isolates of *Penicillium* sp. obtained from infected fruits of different locations were screened to identify the most virulent strain as described by Zahara (2014). The isolate that produced maximum mycelial growth on fruits within 96 hours of inoculation was considered as the most virulent isolate and used for further studies.

Effect of carbon sources on growth of *Penicillium digitatum*:

The Czapek yeast extract media containing 30g of different carbon sources *viz*., dextrose, glucose, lactose, fructose, carboxy methyl cellulose, mannitol and starch substituting the normal carbon source (sucrose) was prepared separately and autoclaved at 1.4 kg/cm² pressure for 20 minutes and allowed to cool. The sterilized and cooled (warm) media was poured into sterilized Petri plates (9 cm) @ 20 ml and allowed to solidify. A 9 mm actively growing culture disc of *Penicillium* sp. cut from 10 days old culture using a sterilized cork borer was placed at the centre of the each petri plate containing the above said solid media separately and incubated for 12 days at room temperature (28 ± 2ºC). Czapek yeast extract media with the normal carbon source (sucrose) served as standard check and three replications were maintained for each treatment.

The Czapek yeast extract broth containing 30 g of different carbon sources *viz*., dextrose, glucose, lactose, fructose, carboxy methyl cellulose, mannitol and starch substituting the normal carbon source (sucrose) was prepared separately without adding agar. Hundred ml broth of each carbon source was distributed uniformly into 250 ml Erlenmeyer conical flasks separately and autoclaved at 1.4 kg/cm² pressure for 20 minutes and allowed to cool. Each flask was then inoculated with 9 mm actively growing Czapek yeast extract culture disc cut from a 10-day old culture of *Penicillium* sp. by means of a sterilized cork borer. The flasks were incubated at room temperature (28 ±2° C) for 20 days for the mycelial dry weight assay and three replications were maintained for each treatment. The Czapek yeast extract broth with the normal carbon source (sucrose) served as standard check. The mycelial dry weight was assessed.

Effect of nitrogen sources on growth of *Penicillium digitatum*

Czapek yeast extract media containing 3.3 g of different nitrogen sources *viz*., ammonium nitrate, urea, potassium nitrate and ammonium chloride substituting the normal nitrogen source (sodium nitrate) was prepared separately and autoclaved at 1.4 kg/cm² pressure for 20 minutes and allowed to cool. The sterilized and cooled (warm) media was poured into sterilized petri plates (9cm) @ 20 ml and allowed to solidify. A 9 mm actively growing culture disc of *Penicillium* sp. cut from 10 day old culture using a sterilized cork borer was placed at the centre of the each petri plate containing the above said solid media separately and incubated for 12 days at room temperature (28 ± 2ºC). CYA media with the normal nitrogen source (sodium nitrate) served as standard check and three replications were maintained for each treatment.

Czapek yeast extract broth containing 3.3 g of different nitrogen sources *viz*., ammonium nitrate, urea, potassium nitrate and ammonium chloride substituting the normal nitrogen source (sodium nitrate) was prepared separately without adding agar agar. Hundred ml broth of each carbon source was distributed uniformly into 250 ml Erlenmeyer conical flasks separately and autoclaved at 1.4 kg/cm² pressure for 20 minutes and allowed to cool. Each flask was then inoculated with 9 mm actively growing CYA culture disc cut from a 10-day old culture of *Penicillium* sp. by means of a sterilized cork borer. The flasks were incubated at room temperature (28 ±2° C) for 20 days for the mycelial dry weight assay and three replications were maintained for each treatment. The CYA broth with the normal nitrogen source (sodium nitrate) served as standard check. The mycelial dry weight was assessed.

Three replications were maintained for each isolate at each treatment. The mycelial dry weight was assessed and for the radial growth of the mycelium was measured

every 2 days after inoculation. The data on all aspects were subjected to statistical analysis of variances (ANOVA) by the procedure given by Gomez and Gomez (1984) using statistical package (SPSS trial version). Prior to statistical analysis the percentage values were arcsine transformed. Wherever, the 'F' test were found significant, critical difference (C.D.) values were worked out at 5 per cent level of probability for comparison of treatment means. The treatment effects were presented by making table of means with appropriate standard error (S.E.) and C.D. value.

RESULTS AND DISCUSSION

Isolation and characterization of *Penicillium digitatum*

The causal agent of green mould disease *Penicillium digitatum* was isolated from the diseased fruits using Czepek yeast extract agar (CYA), and sub cultured by the single hyphal tip method. The isolates from infected fruits were maintained in PDA slants for further studies. The isolated pathogen was tested for pathogenicity by inoculating the pathogen in the healthy oranges. The inoculated oranges were observed for growth of fungal colonies. After four days of inoculation fungal colonies started growing on inoculated oranges. A soft water soaked area developed on the peel on the 4th day which was followed by the development of a circular colony of white mould, up to 4 cm diameter, on the 6th day at 25°C. Green asexual spores (conidia) formed at the centre of the colony surrounded by a broad band of white mycelium on the 7th and 8th day. The fruit rapidly spoiled and collapsed, shrink and mummified on the 10th day. The six various isolates of *Penicillium* sp. obtained from infected fruits of different locations were screened to identify the most virulent strain. The isolate that produced maximum mycelial growth on fruits within 96 hours of inoculation was considered as the most virulent isolate and used for further studies. Among the six isolates tested the isolates 1, 3 and 5 found to infect the fruits in 48 hours and produced symptoms. Other three isolates infected the fruit only after 72 hours. Among the three isolates, isolate 3 was highly virulent as it covered more than 75% of the fruit surface in all the citrus fruits. The three (1,3 and 5) isolates were used for further studies.

Effect of carbon, nitrogen sources on the growth of *Penicillium* sp. in solid and liquid media

Eight carbon sources were tested for growth of *Penicillium* sp.in solid media (Table 1, Plate 1 and Fig. 2) and the results showed significant variation for growth of mycelium. Among the sources, control (sucrose) supported significantly the maximum growth (90.00 mm). This was followed by CMC (71.33 mm), Mannitol (60.67 mm) and Dextrose (60.00 mm) which were all at par with each other and significantly superior over the sources. The minimum growth was recorded in lactose (52.67 mm). Isolate 1 showed maximum growth on sucrose amended medium followed by CMC amended medium. Least growth was recorded in lactose amended medium. Likewise Isolate 5 showed maximum growth on sucrose amended medium and least growth on lactose amended medium.

Growth of *Penicillium* sp.was tested using eight different carbon sources and result indicated significant variation for growth of mycelium (Table 2, Plate 2 and Fig. 2). Among the sources, control (sucrose) supported significantly the maximum growth of isolate 3 by recording mycelial dry weight of 0.98 g followed by CMC (0.72 g) and Mannitol (0.70 g) which were all at par with each other. These three media were significantly superior over all other sources. Lactose recorded the least growth of 0.47 g. Similarly isolate 1 showed maximum growth of 0.97 g in sucrose amended broth and least growth was observed in lactose amended broth (0.44 g). Isolate 5 showed maximum growth of 0.95 g on sucrose amended broth and least growth of 0.41 g on lactose amended broth.

Effect of nitrogen sources on the growth of *Penicillium* sp. in solid and liquid media

The influence of different nitrogen sources on growth of *Penicillium* sp. was studied using five nitrogen sources (Table 3, Plate 3 and Fig. 3). Among the nitrogen sources tested, control (Sodium nitrate) recorded significantly the maximum growth of 90.00 mm for isolate 3. This was followed by potassium nitrate with mycelial growth of 77.00 mm and were at par with each other and significantly superior over other sources. Ammonium chloride recorded least mycelial growth of 62.33 mm for isolate 3. Isolate 1 showed maximum mycelial growth of 89.33 mm on sodium nitrate amended medium and least growth of 59.00 mm on ammonium chloride amended medium. Isolate 5 showed maximum growth of 90 mm on sodium nitrate amended medium and minimum growth on ammonium chloride amended medium.

Among the five nitrogen sources tested for the growth of *Penicillium* sp.in liquid media (Table 4, Plate 3 and Fig. 3), control (Sodium nitrate) was found to be the best by recording significantly the maximum mycelial dry weight of 1.17g by isolate 3.This was followed by potassium nitrate with mycelial dry weight of 0.77g, which was further followed by ammonium nitrate with the mycelial dry weight of 0.71g and urea 0.66g which were at par with each other and significantly superior over other sources. Ammonium chloride recorded the minimum mycelial dry weight of 0.23 g for isolate 3. Isolate 1 showed maximum growth of 1.06 g on sodium nitrate amended broth and least growth of 0.20 g on ammonium chloride amended broth. Isolate 5 showed maximum growth of 1.03 g on sodium nitrate amended broth and least growth of 0.17 g on ammonium chloride amended medium.

Different carbon sources tried in this study revealed that sucrose (control) in CYA medium was the best carbon source which resulted in maximum mycelial growth both in solid and liquid form, for all the three isolates tested. Different nitrogen sources used in this study indicated that sodium nitrate (control) in CYA medium was the best by

Table 1. Effect of different carbon source in solid media on growth of *Penicillium sp.*

Sr.No	Carbon source	Mycelial growth (mm)		
		Isolate 1	Isolate 3	Isolate 5
1	Dextrose	57.33	60.00	56.00
2	CMC (Carboxymethylcellulose)	69.67	71.33	65.00
3	Glucose	53.00	57.33	50.00
4	Mannitol	58.00	60.67	55.00
5	Lactose	50.00	52.67	47.00
6	Fructose	54.00	55.67	49.00
7	Starch	52.33	53.67	48.00
8	Control (Sucrose)	89.67	90.00	89.33
	SEm±	**0.87**	**4.12**	**1.01**
	CD 5%	**2.56**	**12.36**	**2.95**

Table 2. Effect of different carbon source in liquid media on growth of *Penicillium sp.*

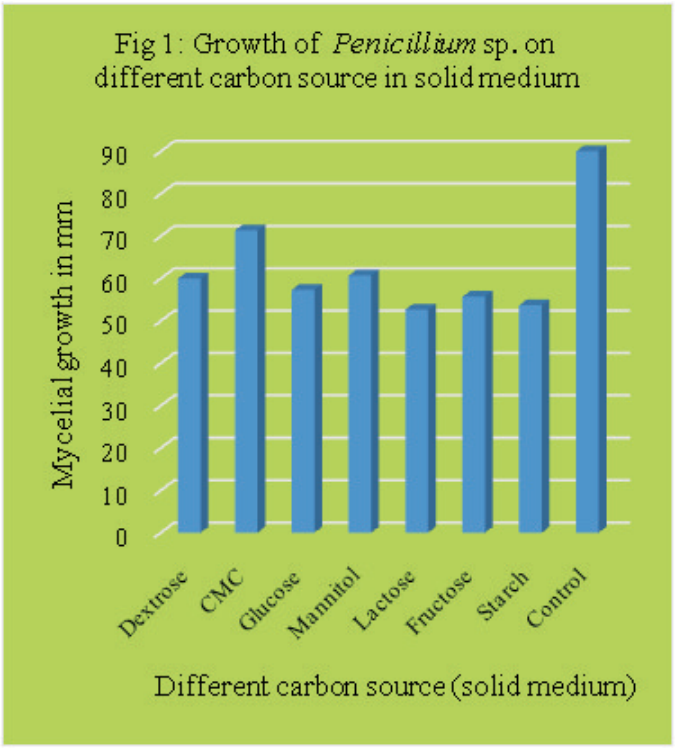
Sr.No.	Carbon sources	Mycelial dry weight (g)		
		Isolate 1	Isolate 3	Isolate 5
1	Dextrose	0.63	0.67	0.61
2	CMC (Carboxymethylcellulose)	0.70	0.72	0.68
3	Glucose	0.54	0.57	0.51
4	Mannitol	0.67	0.70	0.64
5	Lactose	0.44	0.47	0.41
6	Fructose	0.49	0.53	0.47
7	Starch	0.47	0.50	0.44
8	Control (Sucrose)	0.97	0.98	0.95
	SEm±	**0.01**	**0.09**	**0.01**
	CD 5%	**0.03**	**0.28**	**0.03**

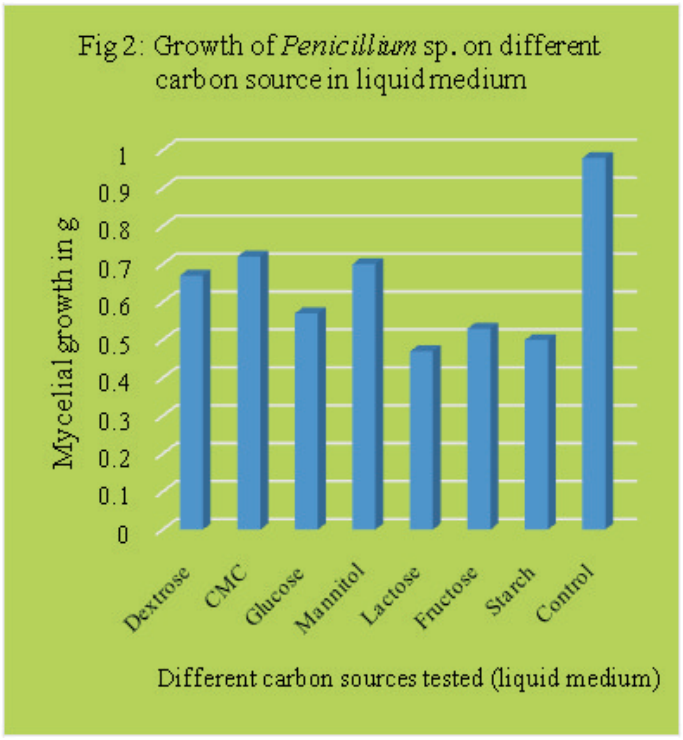
Table 3. Effect of different nitrogen sources in solid media on growth of *Penicillium sp.*

Sr.No	Nitrogen sources	Mycelial growth (mm)		
		Isolate 1	Isolate 3	Isolate 5
1	Potassium nitrate	74.00	77.00	72.00
2	Urea	60.33	64.33	59.00
3	Ammonium nitrate	71.33	75.33	70.00
4	Ammonium chloride	59.00	62.33	59.00
5	Control (Sodium nitrate)	89.33	90.00	90.00
	SEm±	**1.03**	**1.18**	**0.77**
	CD 5%	**3.13**	**3.73**	**2.35**

Table 4.Effect of different nitrogen sources in liquid media on growth of *Penicillium sp.*

Sr.No.	Nitrogen sources	Mycelial dry weight (g)		
		Isolate 1	Isolate 3	Isolate 5
1	Potassium nitrate	0.75	0.77	0.73
2	Urea	0.63	0.66	0.61
3	Ammonium nitrate	0.68	0.71	0.65
4	Ammonium chloride	0.20	0.23	0.17
5	Control (Sodium nitrate)	1.06	1.17	1.03
	SEm±	**0.01**	**0.03**	**0.01**
	CD 5%	**0.03**	**0.11**	**0.04**





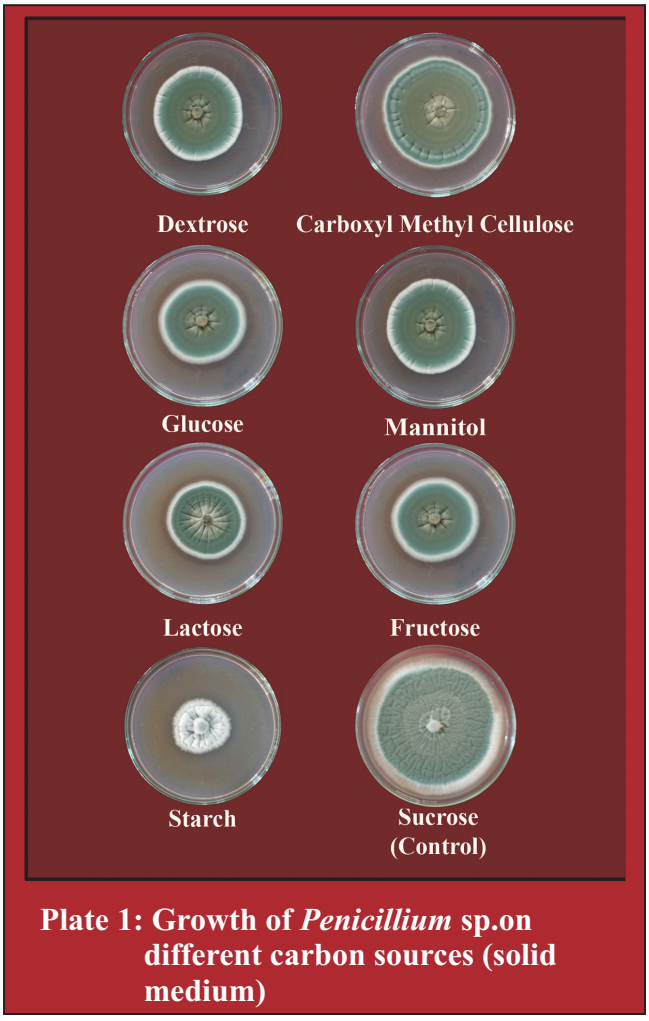


Plate 1: Growth of *Penicillium* sp.on different carbon sources (solid medium)

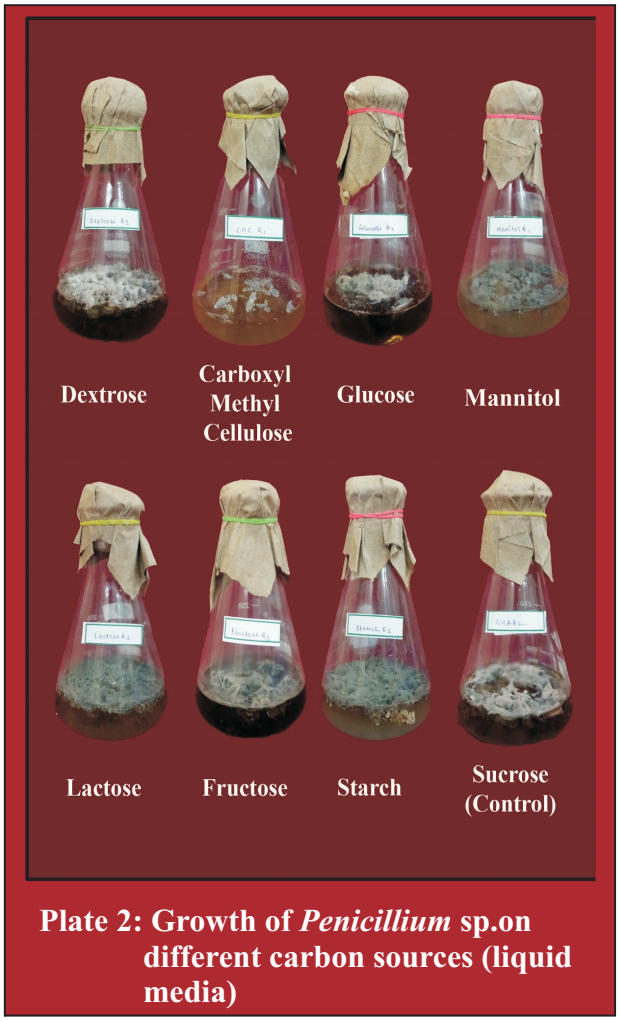
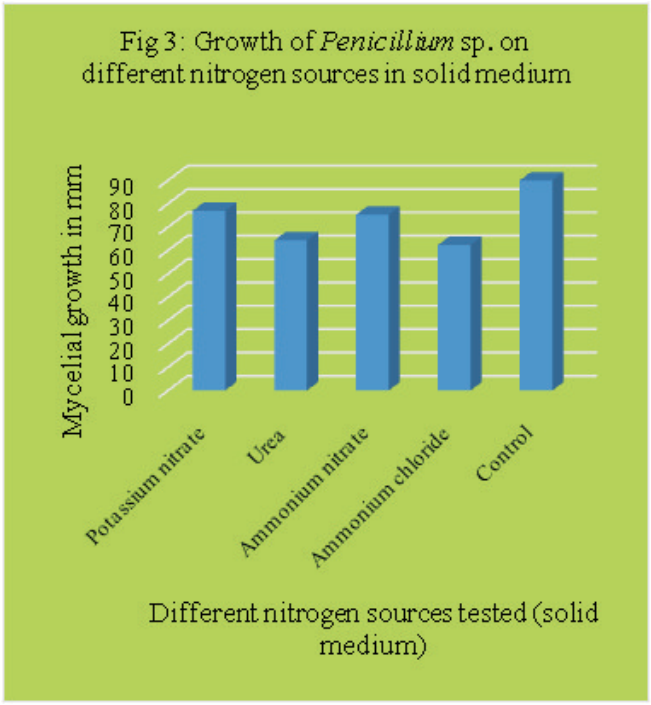
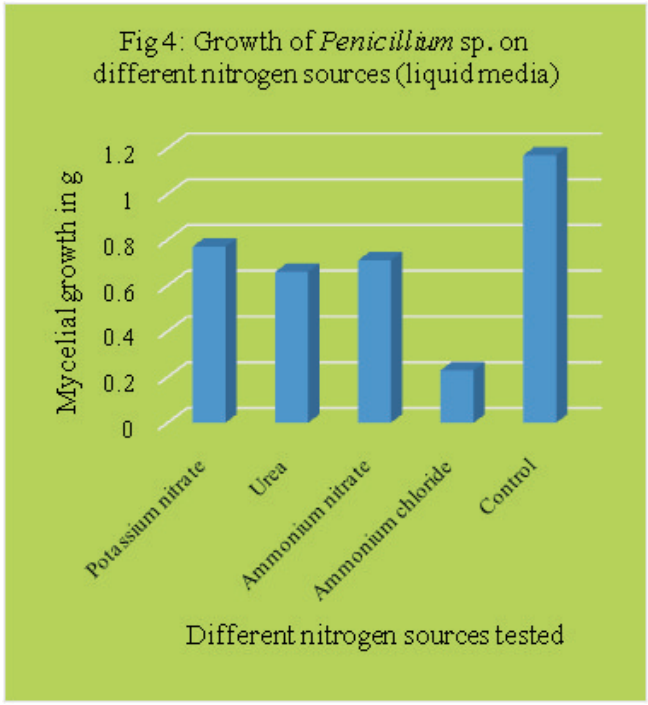


Plate 2: Growth of *Penicillium* sp.on different carbon sources (liquid media)





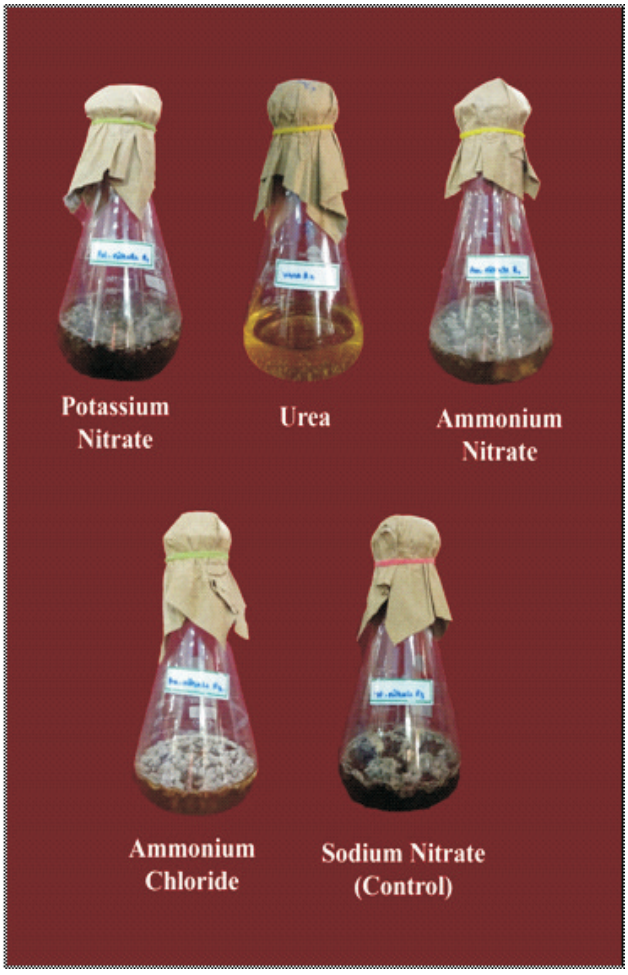


Plate 3: Growth of *Penicillium* sp. on different nitrogen sources (solid medium)

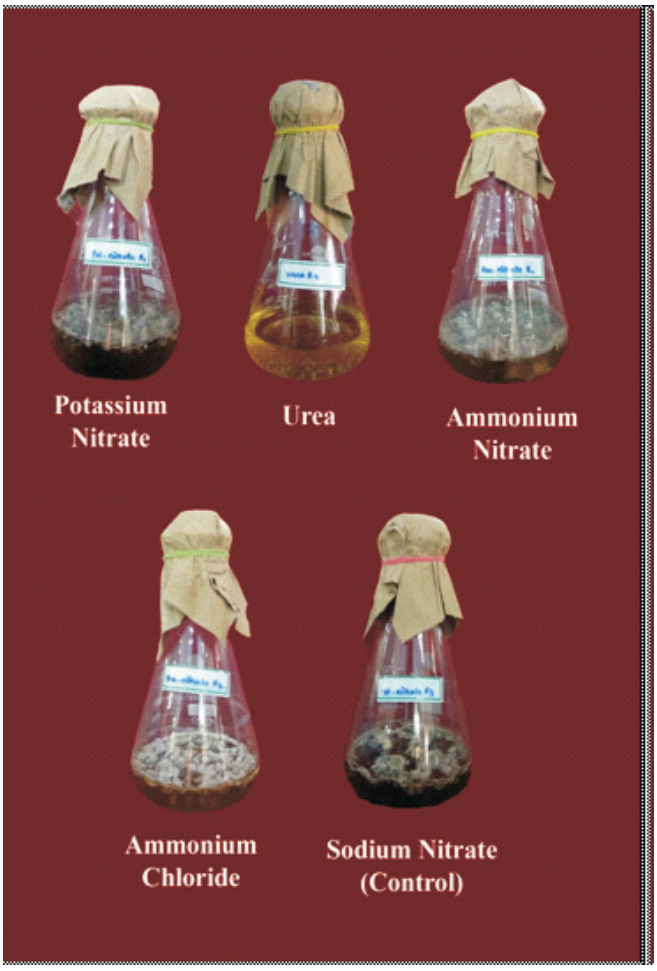


Plate 4: Growth of *Penicillium* sp. on different nitrogen sources (liquid medium)

recording significantly the maximum mycelial growth both in solid and liquid form for all the three isolates tested. In accordance to the present study, Huang (1992) reported that *P. digitatum* spores germinate well in aqueous extract of orange juice or in sugars with presence of phosphate buffer, but very poorly in water with or without buffer or in sugars without buffer. Stange *et al.* (2002) reported that citrus peel extract stimulated growth of *P. digitatum* and *P. italicum* to a much greater degree than *P. expansum*. In contrary to this result Zhou and Yang (2010) reported that the growth of colony was the best with soluble starch as the carbon source and glycine as the nitrogen source on *F. acuminalum.* Khilare and Ahmed (2012) also reported that out of 10 nitrogen compounds tested against *F. oxysporum f.sp. elaeides*; good growth and sporulation were recorded on sodium, ammonium and potassium nitrates, peptone and DL-leucine. Effect of various carbon and nitrogen sources on mycelial growth of *Fusarium* spp. isolated from agricultural fields of Murshidabad was studied by Islam (2015) and reported that among the eight carbon sources used for the study maltose, dextrose, lactose and sucrose were best utilized by all the fungi species. He also reported that among the eight different nitrogen sources used for the growth of the fungi, organic nitrogen compounds were found to be more favourable for mycelial growth of the soil isolates as compared to the inorganic nitrogen compounds and among the inorganic nitrogen sources, sodium nitrate was found most suitable for growth of the isolates.

It is summarized from this study that maximum growth of *Penicillium* sp. occurred in Czapek yeast extract agar (CYA) on solid media and on broth. Among the carbon and nitrogen sources tested, the maximum growth both in solid and liquid media was supported by sucrose and sodium nitrate respectively. The optimum pH for the growth of all the isolates of *Penicillium* sp.was between 6 to 7 and the best pH being 7. Maximum mycelial growth occured at 25°C temperature and 35°C.

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