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Full Length Research Paper

Nutritional factors affecting the antifungal activity of *Penicillium steckii* of mangrove origin

Jyotsnamayee Sabat and Nibha Gupta*

Microbiology Laboratory, Division of Biotechnology, Regional Plant Resource Centre, Bhubaneswar 751 015 (Orissa) India.

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A fungal strain namely, *Penicillium steckii* was obtained from saline environment of mangrove plant *Avicennia marina* and evaluated for antifungal activity in PDA against dieback pathogen of rose. Amendments at the level of different carbon and nitrogen source and their concentrations, inorganic and organic salts resultant into the development of new and modified medium of basal PDA under which our fungi exhibited enhanced level of antifungal activity. Achievement of this medium composition may be useful further in process development to elaborate the antagonistic active principle against plant pathogens.

Key words: Mangrove, avicennia, antifungal, penicillium, dieback, nutrient.

INTRODUCTION

The plants suffer from diseases by agents of both abiotic and biotic nature. The biotic causes are mainly the pathogenic microorganisms. One of the control measures for these diseases is the development of biocontrol agent (Paulitz, 2000; Elad et al., 1998). Microbes in nature can inhibit or arrest the growth of other species (Egrov, 1985). The significant and pronounced form of this antagonism is attributed to the formation of specific metabolites which are popularly known as antibiotics. There are several reports available on the antimicrobial activity and/ or antibiotic-producing capability of microorganisms (Anith et al., 1999; Hariandran et al., 1999; Garcial Kirchner et al., 2000; Somya et al., 2000). Novel secondary metabolites including antibiotics from marine bacteria and fungi are attracting much attention (Ely et al., 2004). Several marine microorganisms have been reported for the production of antimicrobials. Very few reports are available on the antimicrobial microorganisms from mangroves (Bhosle et al., 1999; Christophersen, 1998). Many reports are, however, available on mangrove bacteria (Kathiresan, 2000), but few data on the status of bacteria present in brackish water of Bhitarkanika mangrove ecosystem is available (Gupta et al., 2001; Gupta et al., 2005). Gupta et al. (2001) reported the estuarine Penicillium sp. endowed with antimicrobial properties obtained from mangrove ecosystem. The present study is based on the modification of basal PDA medium for the enhancement in antifungal activity of *Pencillium steckii* active against dieback pathogenic fungi of rose.

*Corresponding author. E-mail: nguc2003@yahoo.co.in. Tel: 91-0674-2557925. Fax: 0674-2550274.

Abbreviations: PDA; potato dextrose agar, **RMI;** relative magnitude of inhibition.

MATERIALS AND METHODS

Microorganisms

Test organisms: *P. steckii* was isolated from rhizosphere soil of mangrove tree species Avicennia marina obtained from Bhitarkanika mangrove ecosystem of Orissa and identified morphologically (Barnett and Hunter, 1972).

Test pathogen: The fungal pathogens used for the present study was isolated from the diseased portion of rose plants grown in the horticultural fields of Regional Plant Resource Centre. This fungal pathogen obtained from the diseased portion of rose caused by dieback and identified as *Nectria cinnabarina* (DB-Dieback) with the help of morphological analysis (Mehrotra et al., 1992; Mehrotra and

Aneja, 1990; Barnett and Hunter, 1972).

***In vitro* antifungal activity test**

The test organisms was tested for antimicrobial activity against rose pathogenic fungi by inoculating them through co inoculation method on agar medium of pH 6.0 and kept under incubation at 30°C for 5 - 7 days. The positive plates which shows the zone around the fungi was measured.

Analysis of inhibition of growth

Zone of inhibition, relative magnitude of inhibition (RMI) and percent inhibition was calculated according to Darokar et al. (1999) and Sing et al. (1999). Zone of inhibition was measured according to mathematical norms, whereas relative magnitude and percentage of inhibition was calculated according to following formulae:

RMI = Area defined by the zone of inhibition including inoculum disc/ area defined by the inoculum disc

% Inhibition = Colony diameter in control - colony diameter in test plate/ colony diameter in control x 100

Effect of nutritional amendments

The potato dextrose agar medium was taken as basal medium for the modification of nutritional amendments. During screening program it was found to be best medium for higher antimicrobial activity showed by *P. steckii.*

Effect of potato extract

The different concentration of potato extract taken was 5, 10, 15, 20, 30, 50, 100 and 150% in place of 25% of basal potato dextrose medium.

Effect of dextrose concentration

The concentration of dextrose taken was 0.5, 1.0, 1.5, 2.5 and 3.0% in place of 2% taken in control set.

Effect of other carbon sources

The different carbon sources taken were sucrose, lactose, starch and mannitol. In each case, the carbon source was taken in 2% w/v and added to the basal PDA medium in place of dextrose.

Effect of combination of carbon sources

In combination with dextrose, other carbon sources were taken viz., sucrose, lactose, starch, and mannitol at the rate of 2% and added to the basal medium.

Effect of sucrose concentration

Sucrose only was found as the best carbon source for this fungal activity. The following concentrations 0.5, 1.0, 1.5, 2.5 and 3.0% (w/v) were taken in a higher order to determine its optimum activity.

Effect of sucrose and glycerol

In combination of sucrose of different concentration, 1% glycerol was added to the medium and observed for antifungal activity under co inoculation method.

Effect of concentration of glycerol

Sucrose 2.5% (w/v) was found to be more suitable. With this concentration of sucrose, different percentage of glycerol was taken that is, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, respectively.

Effect of amino acids

Some amino acids were taken (0.1% w/v) and added to medium. These were L1 arginine, L- glutamic acid, L-methionine, L-tyrosine, L- valine, L- aspargine, lysine, alanine, proline, threonine, phenylalanine, glutamine, and Tryptophan.

Effect of concentration of amino acids

The amino acid in which organism had shown best activity were taken and further added to the medium in percentage of 0.05, 0.1, 0.5,1.0, 1.5 and 2.0% (w/v).

Effect of potassium salts

Two potassium salts that is, KCl and KH_2PO_4, were added to the modified medium at the rate of 0.5% (w/v).

Effect of ammonium and sodium salts

Along with the selected carbon sources in combination (glycerol (%), and amino acid (%)), medium was added with different ammonium salts at the rate of 1%. The salts were ammonium chloride, ammonium ferrous sulphate, ammonium fluoride, ammonium sulphate, ammonium metavendate, ammonium molybdate, ammonium dihydrogen orthrophosphate, ammonium nitrate, ammonium oxalate, ammonium acetate, sodium sulphate and sodium thiosulphate.

Effect of heavy metals and their concentration

To determine the effect of metal ions, $MgCl_2$ and $MnSO_4$ were taken in different concentration that is, 0.01, 0.03, 0.05, 0.07 and 0.1% in to the modified medium.

Effects of hormones and their concentration

To determine the effect of plant growth hormones, naphthyl acetic acid, benzyl adenine, kinetin and gibberellic acid in concentration of 10, 15, 20 and 25 ppm, respectively, were taken.

RESULTS

Studies carried out to determine the effects of different

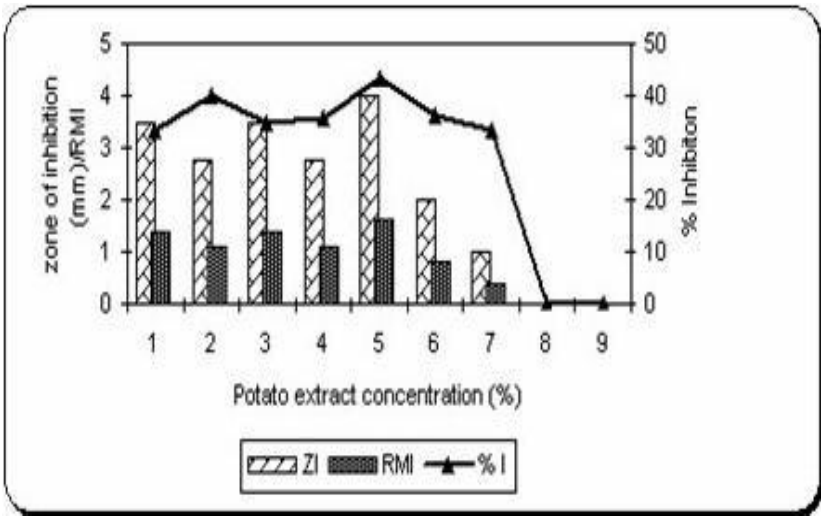

Figure 1. Effect of potato extract. Medium compositions - Dextrose (2%) + Potato extract [1 = 5%, 2 = 10%, 3 = 15%, 4 = 20%, 5 = 25%, 6 = 30%, 7 = 50%, 8 = 100% and 9 = 150%].

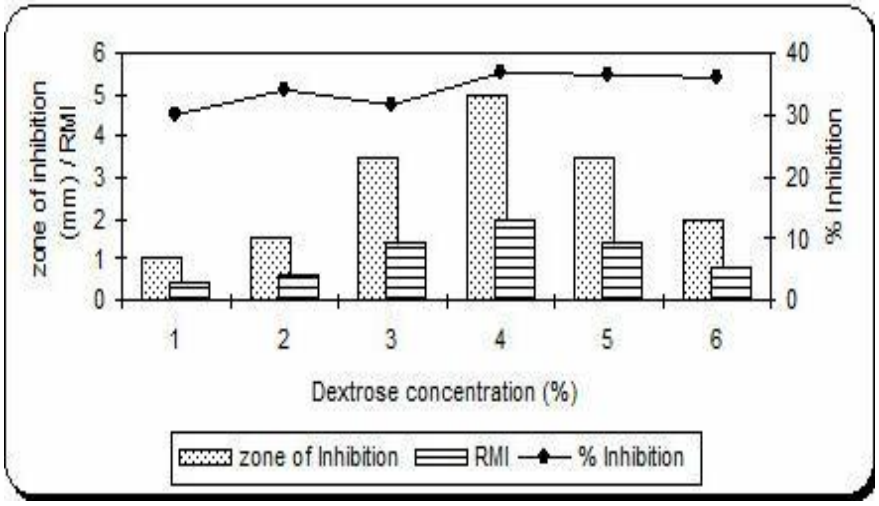

Figure 2. Effect of dextrose. Medium compositions - Potato extract (25%) + Dextrose concentration [1 = 0.5%, 2 = 1.0%, 3 = 1.5%, 4 = 2.0%, 5 = 2.5% and 6 = 3.0%].

nutrients upon the antifungal activity of *P. steckii* have exhibited some significant results to elucidate its nutritional preference and metabolism related to antagonistic active principle. The results obtained are described as follows:

The effect of potato extract

The media taken was basal type that is, potato dextrose agar. The data showed that the organism preferred 25% potato extract for giving higher zone of inhibition to the other concentration of potato extract taken. The inhibition zone and RMI recorded was 4.5 and 1.6 mm, respectively (Figure 1). In this experiment, 25% potato extracts showed 43.33% inhibition that much higher as compared to other concentrations of potato extract taken.

The effect of dextrose concentration

Out of six different concentration of dextrose tested, the highest zone of inhibition was observed in 2% dextrose that is, 5 mm (Figure 2). In other concentrations of

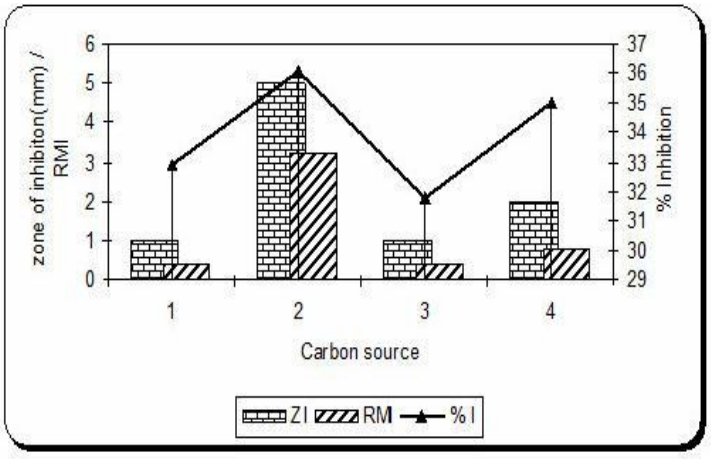

Figure 3. Effect of carbon sources. Medium compositions - Potato extract (25%) + other carbon sources (2%), 1 = Lactose, 2 = Sucrose, 3 = Starch and 4 = Manitol.

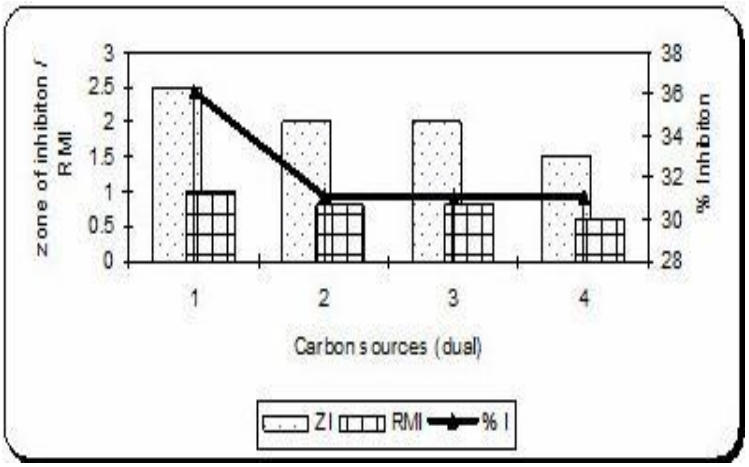

Figure 4. Effect of dextrose and other carbon sources. Medium compositions - Potato extract (25%) + Glucose (2%) + other carbon sources (2%), 1= Lactose, 2 = Sucrose, 3 = Starch and 4 = Manitol.

dextrose, this fungus did not produce good inhibition zone. Therefore, 2% dextrose was selected for further studies (Figure 2). Relative magnitude of inhibition was higher in this concentration of dextrose, whereas similarvalue of percentage inhibition was observed in experimental set up in which dextrose was used in 2, 2.5 and 3.0% concentration.

Effect of other carbon sources

Out of 4 carbons sources taken, the best result obtained was with sucrose (Figure 3). In PDA containing 25% potato extract, 2% sucrose exhibited 5 mm zone of inhibition. It was higher as compared to previous experiments. This has also showed the highest RMI that is, 3.2 and percentage inhibition of 36.1, which was quite higher than other carbon sources used.

Effect of other carbon sources along with dextrose

Four carbons sources (Lactose, sucrose, starch and mannitol) were taken separately in combination with 2% dextrose + 25% potato extract. These carbon sources exhibited poor inhibition against test pathogen than the only carbon source used (Figure 4). Lactose exhibited higher zone of inhibition, RMI and percentage inhibition

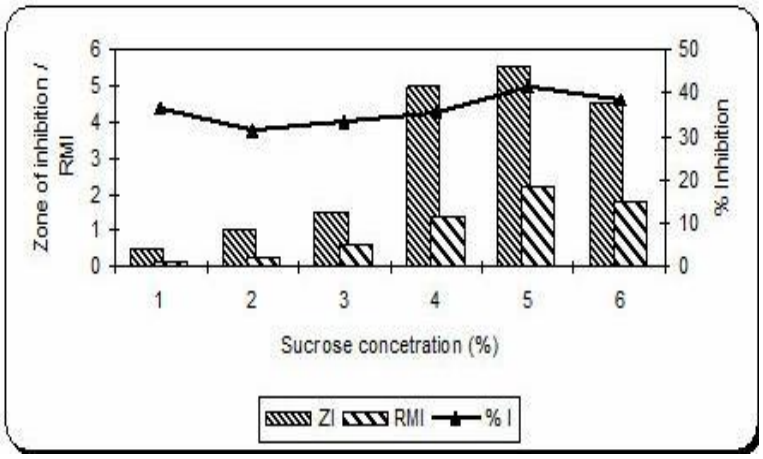

Figure 5. Effect of sucrose, Medium compositions - Potato extract (25%) + Sucrose concentration [1 = 0.5%, 2 = 1.0%, 3 = 1.5%, 4 = 2.0%, 5 = 2.5% and 6 = 3.0%].

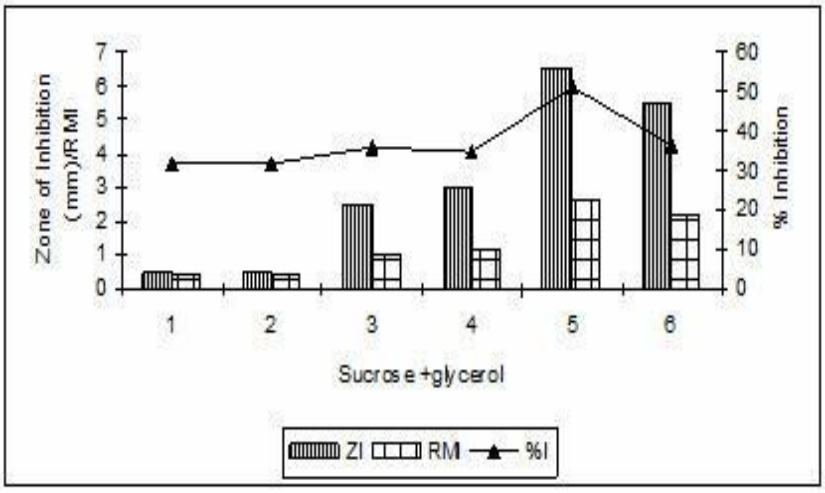

Figure 6. Effect of sucrose and glycerol. Medium compositions - Potato extract (25%) + Glycerol (1%) + Sucrose concentrations [1 = 0.5%, 2 = 1.0%, 3 = 1.5%, 4 = 2.0%, 5 = 2.5% and 6 = 3.0%].

but comparatively it was lower than the sucrose used singularly. Therefore, only sucrose was selected for further modification of the medium.

Effect of concentration of sucrose

Sucrose was taken in different concentration along with 25% potato extract (Figure 5). Concentration wise, the best concentration 2.5% exhibited 5.5 mm zone of inhibition which is higher among the all previous experimental sets taken. The two concentrations of sucrose that is, 2 and 2.5% gave higher zone of inhibition and RMI. However, at 2.5% concentration of sucrose highest level of inhibition on growth of pathogenic fungus was observed. Therefore, along with 25% potato extract, only sucrose was taken as carbon source in concentration of 2.5%.

Effect of glycerol

In different concentration of sucrose, glycerol was taken at rate of 1% into the modified medium (Figure 6). Again, in the 2.5% concentration of sucrose 1% glycerol showed positive effect and gave 6.5 mm of inhibition zone.

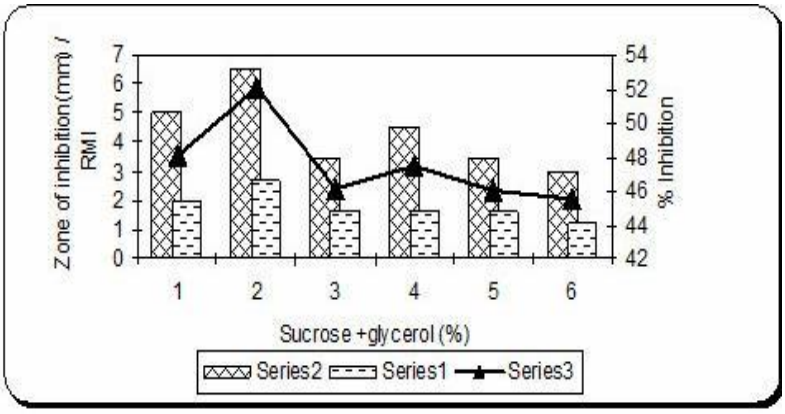

Figure 7. Effect of glycerol concentration. Medium compositions - Potato extract (25%) + Sucrose (2.5%) + Glycerol [1 = 0.5%, 2 = 1.0%, 3 = 1.5%, 4 = 2.0%, 5 = 2.5% and 6 = 3.0%].

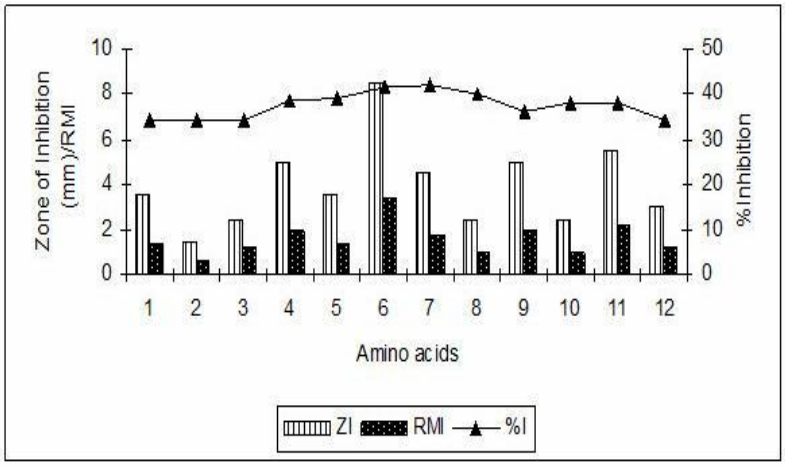

Figure 8. Effect of amino acids. Medium composition: Potato extract 25% + Sucrose (2.5%) + Glycerol (1%). 1 = no amino acid, 2 = L- arginine, 3 = L-Glutamic acid, 4 = L- Methionine, 5 = L- Tyrosine, 6 = L- lysine, 7 = L-asparagine, 8 = Lvaline, 9 = alanine, 10 = proline, 11 = threonine, 12 = phenylalanine, 13 = glutamine and 14 = tryptophan.

Results exhibited the positive effect of combination of sucrose and glycerol. The enhancement in the RMI and percentage inhibition from 2.2 - 2.6 and 41.66 - 51.11, respectively also suggested the usefulness of glycerol in combination with sucrose for the further modification of medium.

Effect of concentration of glycerol

The effect of 6 different concentrations of glycerol into the medium was taken for antifungal test (Figure 7). It exhibited good response at 1% glycerol and results showed 6.5 mm zone of inhibition in this combination. RMI and percentage Inhibition had also enhancement. Therefore, 1% glycerol was selected.

Effect of amino acids

Total 13 amino acids were tested for the further medication of medium in 0.1% concentration (Figure 8). In case of all amino acids tested, inhibition of growth of test pathogen could be observed but significant change in inhibition was observed with lysine that exhibited 8.5 mm zone of inhibition. Though only 41.66% of inhibition could be observed in this experimental set, very high value of RMI (3.4) was observed with addition of lysine into the

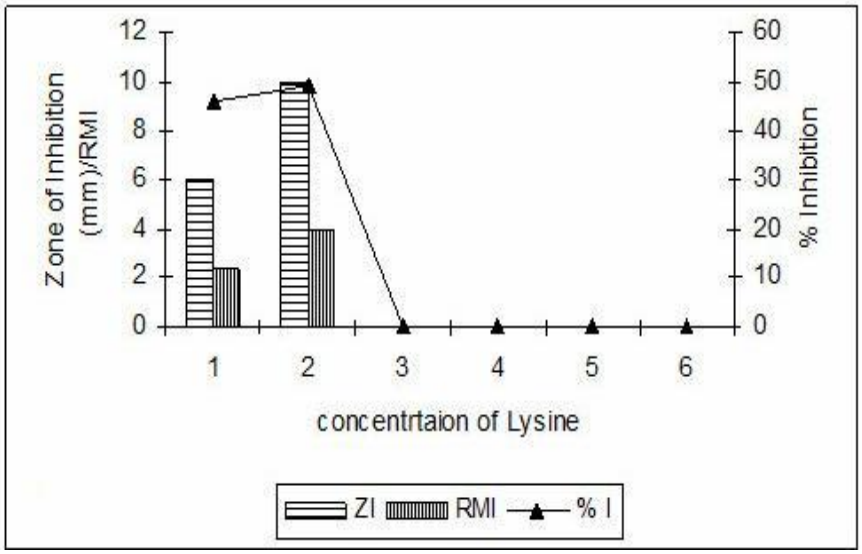

Figure 9. Effect of concentration of amino acid. Medium composition: Potato extracts 25% + Sucrose (2.5%) + Glycerol (1%). Lysine (1 = 0.05%, 2 = 0.1%, 3 = 0.5%, 4 = 1.0%, 5 = 1.5% and 6 = 2.0%).

medium. Therefore, lysine was selected.

Effect of concentration of lysine

6 different concentrations of lysine were tested in combination with the modified medium (Figure 9). The results obtained have shown that 0.1% concentration exhibited good inhibition towards growth and produced 10 mm zone around the growing colony of test pathogen. Lysine in low concentration of 0.05% also gave good results but the addition of 1.0% lysine to the modified medium also relative magnitude of inhibition up to 4 that made the basis of lysine for further selection.

Effect of metals

Metal salts used in this study were $MgCl_2$ and $MnSO_4$ in different concentrations (Figure 10). The effect of both metal salts was negative upon the antifungal activity of fungi. In the presence of metal ions, test fungi could not exhibit a good inhibition towards growth of dieback fungi. Therefore, no metal salt was selected further.

Effect of hormones

Four plant growth-promoting hormones were tested into the modified medium in different concentrations (Figure 11). There was a negative effect of all the four hormones tested as reduction in inhibitory activity of *Penicillium* sp. as compared to control (with out hormones) was observed. However, addition of hormones into the modified medium exhibited the antifungal activity that was not higher as compared to control. Therefore no plant growth hormone could be selected for the further modifications. All of them affected reduction in the inhibition zone indirectly antifungal activity of *Pencillium* sp.

Effect of ammonium, sodium and potassium salts

There was no effect on antifungal activity of *P. steckii* rather, growth of the organism was retarded as compared to previous modification experimental sets. Therefore, no such salt were be selected for the further modification of the medium.

Development of modified medium

All these experimental sets resulted into the development of modified medium that contained 25% potato extract, 2.5% sucrose, 1% glycerol, 0.1% lysine, pH 5 and incubation period 6 days in solid condition. In original and normal medium of PDA (pH 5.0), the fungi produced 4.5 mm zone of inhibition where as in the modified medium it exhibited 10 mm zone of inhibition. In the modified medium developed through this experimental study, results have shown quite enhanced antifungal activity of fungus towards dieback pathogen.

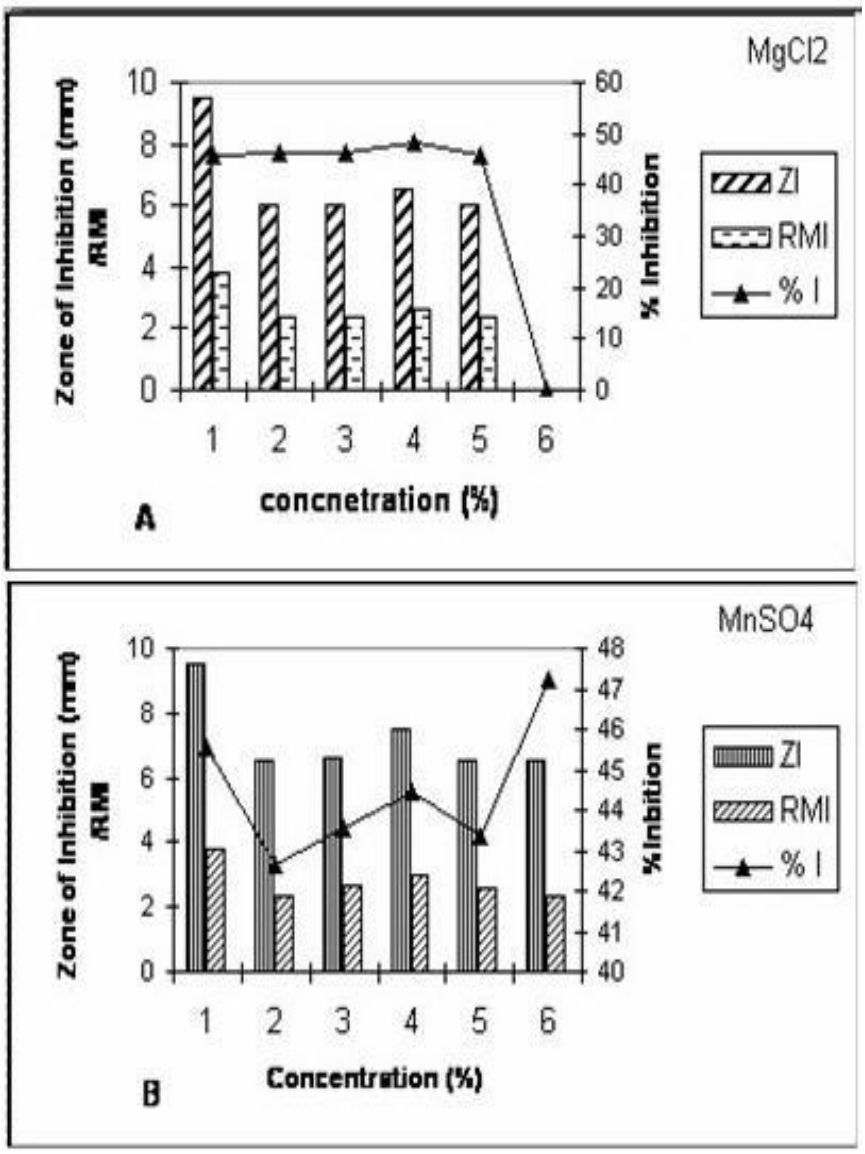

Figure 10. Effect of concentration of metals. Medium composition: Potato extracts 25% + Sucrose (2.5%) + Glycerol (1%) + Lysine 0.1%. Metal concentration: 1 = 0, 2 = 0.01%, 3 = 0.03%, 4 = 0.05%, 5 = 0.07 and 6 = 0.1%.

DISCUSSION

The experimental studies carried out to evaluate cultural requirement for *P. steckii* have provided good information on effects of test parameters upon antifungal activity on solid state of the medium. Nutritional modifications such as carbon source, nitrogen sources, etc, have affected antifungal activity of *P. steckii* variously. The medium taken was basal type because in previous studies this organism had shown preference for the Potato dextrose agar (PDA) of pH 5.0 with respect to antifungal activity towards dieback pathogenic fungi. This also gets further corroborated with the facts reported by several workers who obtained PDA as the best medium for growth of some fungi (Selvan and Setharaman, 2003; Leon et al., 1999). It also showed the preference of this fungus for natural substrate and carbon source especially starch. Standardization of potato extract requirement confirms the standard medium composition given earlier (Aneja, 1993). It appeared that the fungi needed a particular concentration of starch in the medium, though it might be in the form of natural substrate.

In standard medium of PDA, 2% dextrose is one of the components. Similarly, in the present study, the test

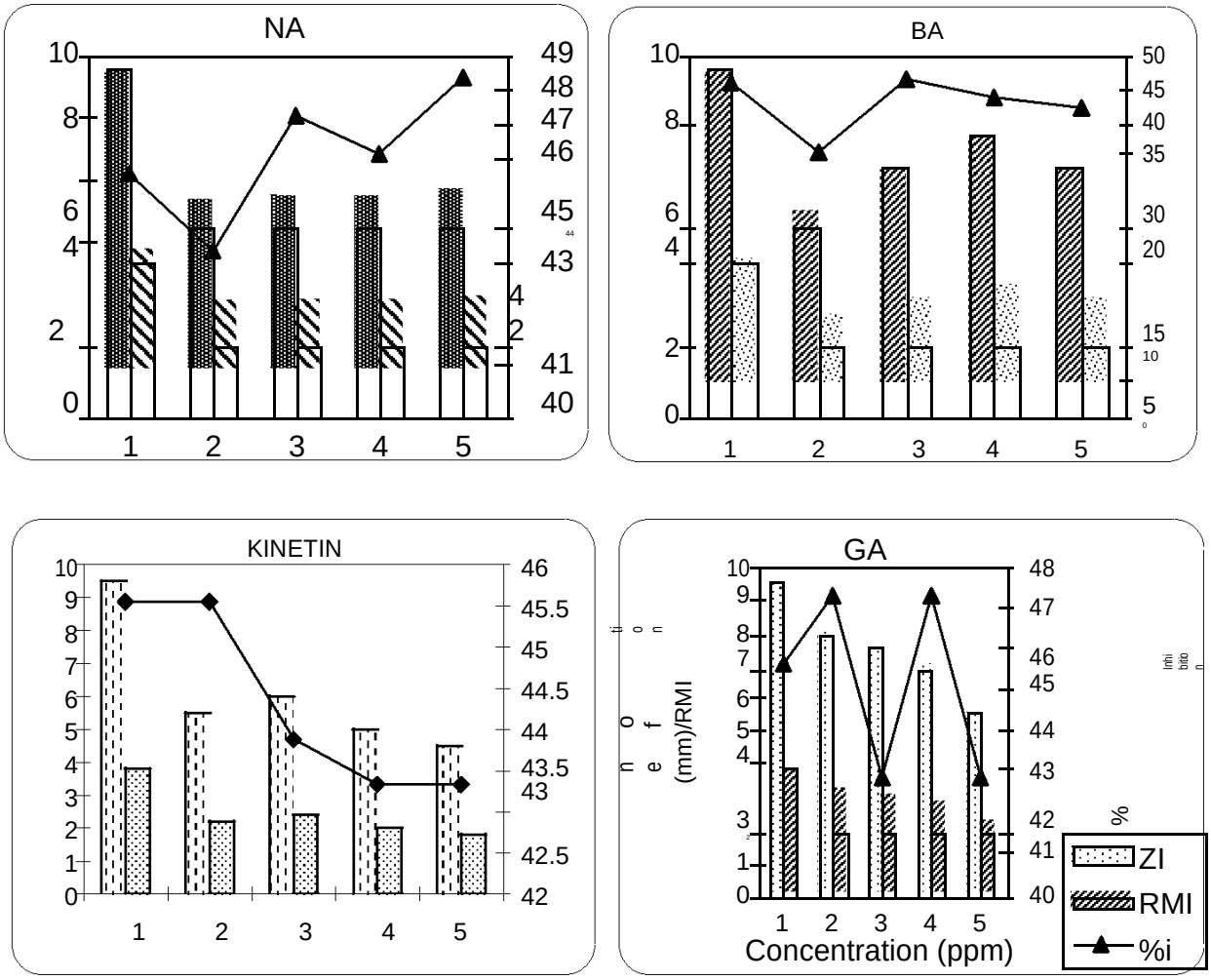

Figure 11. Effect of concentration of hormones. Medium composition: Potato extracts 25% + Sucrose (2.5%) +Glycerol (1%) +Lysine (0.1%). Concentration of hormones: 1 = 0; 2 = 10 ppm; 3 = 15 ppm; 4 = 20 ppm; 5 = 25 ppm.

organism preferred 2% dextrose. It is reported that glucose containing media sometimes adversely affects the growth of organisms indirectly to metabolisms (Carter and Bull, 1969; Siddiqui and Shaukat, 2002). The present fungi utilize sucrose as the primary source of carbon. Preference of test fungi for sucrose showed that such nutrients continue to support biomass for long periods of incubation than dextrose. (Borrow et al, 1961; Costa et al., 2002). This was also supported by Monga (2001) who had used this approach to analyse the effect of carbon and nitrogen sources on growth of Trichderma sp. In general, sucrose was found to be best for sporulation. The effect of other carbon sources like lactose, starch, and mannitol could not be found to be suitable as a factor enhancing antifungal activity of test fungi though some of them are reported as best for growth of several fungi (Simley et al., 1967; Varshney, 1981).

Inclusion of glycerol in appropriate quantities with the growth medium enhances the antimicrobial activity (Siddiqui and Shaukat, 2002; Ismet et al., 2004). The addition of glycerol (1%) enhanced the antifungal activity of our fungi. The postassium salts had no effects on the fungal activities on laboratory conditions. Similarly, different salts of ammonium did not show any effect upon the antifungal activity of this fungus, although several ammonium and potassium salts were reported to be effective in enhancing growth and metabolism of fungi (Sood, 1990). Amino acids were reported as good sources of nitrogen and accelerate the biomass production of fungi. Among them alanine, glycine, arginine and aspartic acid are the best source for the growth of bioagents (Pateman and Kinghorn, 1976; Monga, 2001). In similar way, in the present study amino acids exhibited their positive effects upon such secondary metabolisms of our organism and lysine produced best inhibition zone against pathogenic fungi of dieback of rose.

Some growth factors that affect the metabolic efficiency of fungi are trace elements, vitamins and hormones. The

plant growth hormones and metal ion- containing salts are quite well known for their positive impact on the physiology of certain microbes (Sood, 1990). Some of such compounds were evaluated for their effects on antifungal activity of *P. steckii*. There were effects of all plant growth hormones tested on the antifungal activity of fungi and it is quite higher in comparison with the experimentation done for the carbon sources and their concentration. There was no relation of their effects to their respective concentrations. The growth promoting and retarding effects of metal salts are also reported. The effects of both metal salts were negative upon the antifungal activity. To this effect, it was not worthy of incorporation of these metals and hormones into the medium.

In the present study that was based on the modification process of cultural medium required for the enhancement of production of secondary metabolite having antifungal activity against dieback pathogen of rose and produced by *P. steckii,* good production of this biocontrol agent on laboratory scale and the potential of scaling up the process is encouraged. This study stands to be of much importance due to its source (high salinity zone of Bhitarkanika mangrove ecosystem) . Secondly, the optimization of cultural conditions may be used as a key factor in fermentation process (that is, as a prerequisite to achieve desired level of product formation at industrial level).

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