Indian Journal of Chemical Technology
Vol. 30, November 2023, pp. 797-804
DOI: 10.56042/ijct.v30i6.6541

Exploration of antimicrobial and antifungal compounds in *Clonostachys rosea* using gas chromatography - mass spectrometry

Gowrisri Nagaraj[1], Kannan Rengasamy[1]*, Raguchander Thiruvengadam[1], Muthusamy Karthikeyan[1], Varanavasiappan Shanmugam[2] & Swarnakumari Narayanan[3]

[1]Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu- 641 003, India

[2]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu- 641 003, India

[3]Department of Nematology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu- 641 003, India

*E-mail: kannanar2004@gmail.com

Received 11 August 2022; accepted 25 August 2023

The GC-MS analysis of secondary metabolites of *Clonosatchys rosea* (*C. rosea*) reveals the presence of antimicrobial and antifungal compounds which is essential attire for any biocontrol agent to be commercialized. Experimental evidence showing importance of carbon and nitrogen sources on growth and sporulation of *C. rosea* (TNAU CR 01) has been undertaken. Among eleven carbon sources tested, dextrose has been found to be effective followed by sucrose in improving the mycelial growth and sporulation of the fungus. Casein has been identified as a significant nitrogen source responsible for the improvement in fungal growth and sporulation followed by potassium nitrate, phenylalanine and sodium nitrite. When considering the future commercialization of novel biocontrol agents like *C. rosea*, the current study will be useful in understanding the importance and choice of nutritional sources required for their growth and development of biocontrol agents and chemical analysis of secondary metabolites will be helpful in understanding their mode of action against phytopathogens.

Keywords: Carbon sources, *Clonostachys rosea*, GC-MS, Nitrogen sources, Secondary metabolites

Pathogens affect numerous economically important crops by causing diseases. The majority of diseases are challenging for traditional fungicides to manage[1]. In order to reduce the use of chemical pesticides, efforts have been made to isolate and develop indigenous biocontrol agents against a variety of soil-borne plant pathogenic fungus. For diseases caused by phytopathogens, biological control provides an affordable, ecologically sound alternative to chemical pesticides.

Nutrients are essential elements for any organism to be viable, survive and to be sustained. They are utilized in larger amounts for biosynthesis and energy release[2]. An in-depth understanding of the nutritional needs of microorganisms is essential for their elite cultivation. The metabolically active macro elements, such as carbon, oxygen, hydrogen, nitrogen, sulphur, and phosphorus, are essential parts of carbohydrates, lipids, proteins, and nucleic acids and play a direct or indirect role in host-pathogen interactions as well as self-defense and perpetuation mechanisms[3].

The fungal species of *Clonostachys rosea* (*C. rosea*) are found all over the world. They mainly live as saprotrophs, destructive mycoparasites, lichenicoles, or dwellers of recently dead trees and decomposing leaves[4]. This widespread fungus species is a potential and efficient biological control agent that is abundantly observed naturally in agricultural soils[5]. To commercialize them as biofungicide, appropriate cultural techniques and biomass production should be established and the best conditions for spore production also need to be identified. In the present study, the effect of carbon and nitrogen sources on the growth and sporulation of *C. rosea* was discussed and their secondary metabolites were analysed using Gas chromatography - Mass Spectrometry for the presence of antimicrobial and antifungal compounds to support their commercialization as an effective bioagent. To the best of our knowledge, no previous studies were focused on growth conditions of *C. rosea* in India, thereby making ours first in line.

Experimental Section

Clonostachys rosea strain

The *C. rosea* strain TNAU CR 01 was isolated from rhizosphere soil of carrot collected from cultivable fields in Nanjanad, Ooty. The strain TNAU CR 01 has the strongest antagonistic property and is most eligible for promoting as an effective biocontrol agent. The fungal antagonist was maintained in potato dextrose agar medium and stored at 4°C for further studies.

Effect of carbon sources on growth and sporulation of *C. rosea*

Eleven different carbon sources *viz.,* Arabinose, Carboxy methyl cellulose, Chitin, Chitosan, Dextrose, Mannitol, Pectin, Sodium acetate, Sorbitol, Starch and Sucrose were added to potato dextrose medium after autoclaving (@121°C with 15 psi for 20 min). Streptomycin sulphate at 500 ppm was added to the autoclaved medium to avoid other bacterial contamination. A fungal disc of 4 mm diameter was plugged out from the edge of actively growing 7 days old culture of *C. rosea* (TNAU CR 01) and placed in the centre of 50 mm sterile plastic Petri plates. All the inoculated plates were maintained at 25 ± 2°C for about 15 days. Each treatment was replicated thrice.

Effect of nitrogen sources on growth and sporulation of *C. rosea*

Eleven different nitrogen sources *viz.,* Ammonium nitrate (2 g/L), Ammonium sulphate (2 g/L), Arginine (2 g/L), Ascorbic acid (2 g/L), Asparagine (2 g/L), Casein (2 g/L), Phenyl alanine (2 g/L), Potassium nitrate (2 g/L), Tris HCL (2 g/L), Sodium nitrate (2 g/L) and Sodium nitrite (2 g/L) were added to CZA medium (Czapek Dox Agar) containing Sucrose (30 g/L), Sodium nitrate (2 g/L), Dipotassium phosphate (1 g/L), Magnesium sulphate (0.5 g/L), Potassium chloride (0.5 g/L), Ferrous sulphate (0.01 g/L), Agar (15 g/L) and Final pH (at 25°C) 7.3±0.2 after autoclaving and the fungus was inoculated as mentioned above. The standard concentration of Sodium nitrate (2 g/L) in CZA medium was replaced by above mentioned ten other different nitrogen sources to test their efficacy on growth and sporulation of *C. rosea*.

Determination of mycelial growth and sporulation of *C. rosea*

At two days interval (3, 5, 7, 9 and 12 DAI) the plates from each treatment were taken for the measurement of mycelial growth and the spore production was also observed under phase contrast microscope (Labomed) until 12 days after incubation. Based on the amount of sporulation, they were classified as good, medium and poor sporulation under each treatment.

Extraction of non-volatile crude metabolites from *C. rosea*

C. rosea isolate (TNAU CR 01) was selected for crude metabolite production and grown in 250 mL conical flask containing Potato Dextrose Broth. The flask was incubated at 25°C for 12 – 15 days. Then the cultures were drained through two coats of filter paper. To the extracted culture filtrate equal volume of ethyl acetate was added and kept in shaker (150 rpm) for overnight incubation. Using separating funnel, the ethyl acetate coat was detached. The collected upper phase of the ethyl acetate coat was evaporated under rotary evaporator and concentrated to dryness and the dried blanket was dissolved in 1 mL of HPLC grade methanol.

Efficacy of crude metabolities from *C.rosea* against *Fusarium oxysporum* f.sp. *lycopersici*

The crude metabolite extracted from *C. rosea* was tested for its antimicrobial activity against *Fusarium oxysporum* f.sp. *lycopersici* (F1 isolate) using agar well diffusion assay. About four different concentrations of crude extracts *viz*., 250, 500, 750 and 1000 ppm of *C. rosea* were tested against the fungal pathogen. Around 75 µL of extract was added to the well 1 cm away from the edge on four sides of the Petri plate. A nine mm fungal disc was placed in the centre of the Petri plate. All the treated plates were incubated at 25 ± 2°C until the fungal mycelium covers the control plate with sterile water poured in each well.

GC-MS analysis of non-volatile metabolities of *C.rosea*

The non- volatile crude metabolities collected from *C. rosea* isolate was subjected to GC-MS analysis, for which the trubo mass-gold-perkin-Elmer detector was used. The Clarus SQ 8C Gas Chromatography – Mass Spectrometer from Perkin Elmer instrument for the above purpose was set as follows, Injector port temperature set to 220°C, Interface temperature set to 250°C, source kept at 220°C. The oven temperature programmed as 75°C for 2 min, 150°C @ 10 °C/min, up to 250°C @ 10 °C/min. Split ratio set as 1:12 and the split less injector mode was used. The DB-5 MS capillary standard non-polar column with 0.25 mm OD × 0.25 µm ID × 30 meters dimension was used. Helium at 1 mL/min was used as the transporter gas. The source was maintained at 220°C and $4.5e^{-6}$ motor vacuum pressure. The ionization energy was -70 eV. NIST MS Search 2.2v contains more than five lakh references. Therefore, Interpretations were made using the database of National Institute of Standard and Technology (NIST14 software).

Statistical analysis

The data were statistically analysed using the SPSS version 16.0 (Statistical Package for the Social Sciences) developed by Norman. H. Nie, Dale H. Bent, and C. Hadlai Hull in 1968. The data were subjected to analysis of variance (ANOVA) at two significant levels (P<0.05 and <0.01) and means were compared by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Effect of carbon sources on growth and sporulation of *C.rosea*

Carbon is responsible for half of the dry weight of the fungal cells, which clearly defines the pivotal role of carbon sources within the cells. Nitrogen is a vital component for the growth and metabolic activity of fungi to metabolize varied nitrogen sources in order to colonize wide environmental niches and endure nutrient scarcity. The effect of growth and sporulation of *C. rosea* has been less explored which is of paramount importance to achieve large amount of biomass production to commercialize them as a potential biocontrol agent. In the present study, the effect of different carbon sources on the growth and sporulation of *C. rosea* was analyzed.

The *C. rosea* grows well in almost all the eleven carbon sources tested. The growth of the mycelium and the spore production was significantly higher in dextrose amended potato medium covering 4.9 cm (mean radial growth) followed by sucrose with 4.7 cm (mean radial growth) at 15 days after incubation (Fig. 1). The least growth and sporulation of *C. rosea* was recorded in carboxy methyl cellulose with 3.0 cm (mean radial growth) but surprisingly with good sporulation was recorded (Table 1). Based on the current findings, it is clearly evident that dextrose served as a growth stimulant for the fungus under study along with sucrose which is an excellent source for enhancing spore production. Our findings were supported by works that indicated the better growth and sporulation of *Trichoderma viride* (*T. harzianum*) when dextrose was used as a carbon source[6]. Similarly, the increased biomass production and sporulation of *T. harzianum* was reported when honey followed by dextrose were used as a carbon source[1]. The highest production of *Fusarium* macroconidia in basal medium amended with carboxy methyl cellulose as a carbon source was observed[7] which is on par with our findings.

Effect of nitrogen sources on growth and sporulation of *C. rosea*

All the eleven nitrogen sources tested were found to be increasing the growth and sporulation of

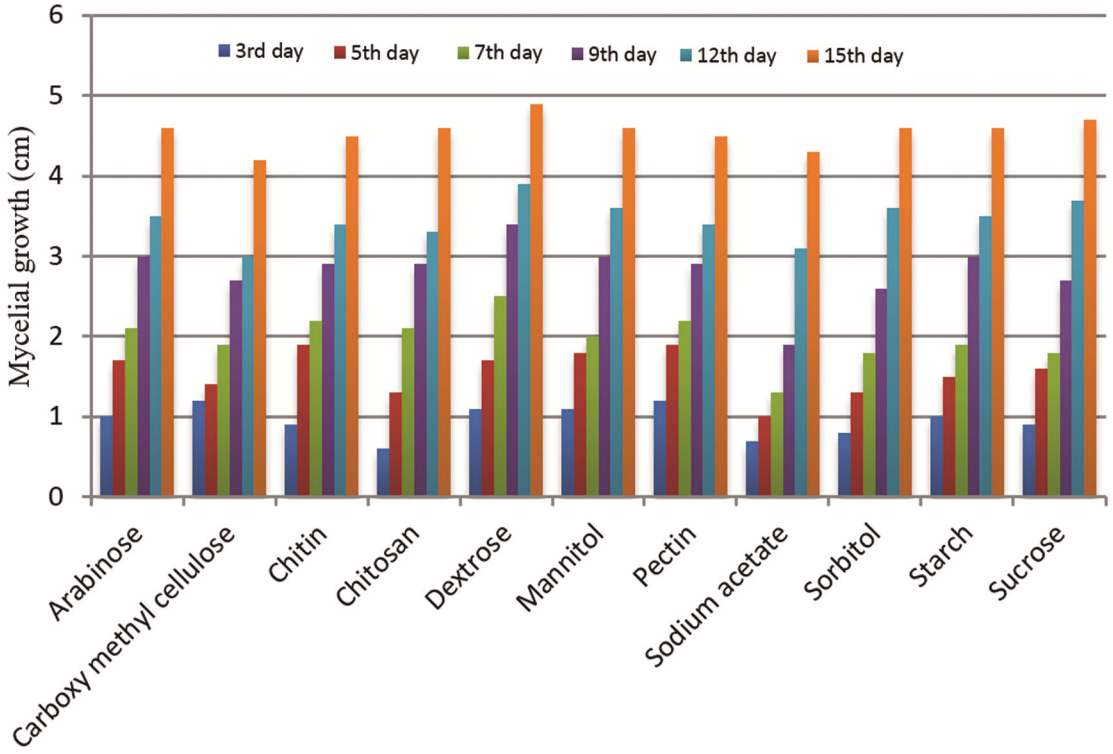
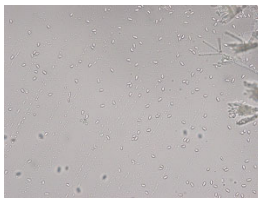
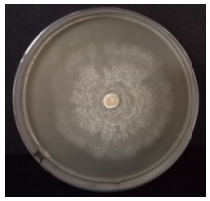
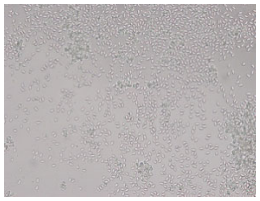
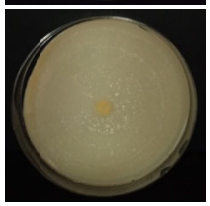
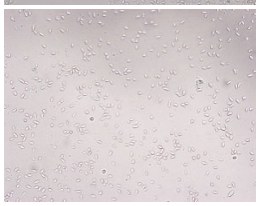
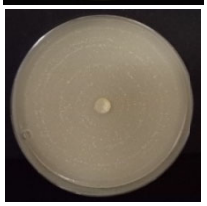
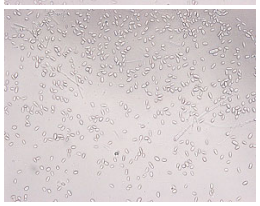
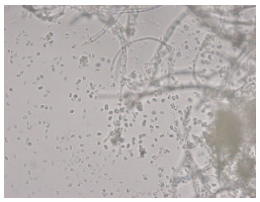
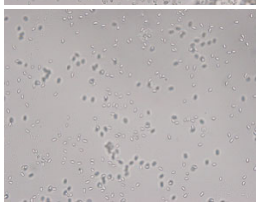
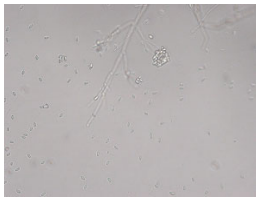
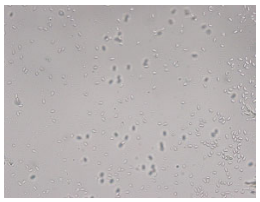


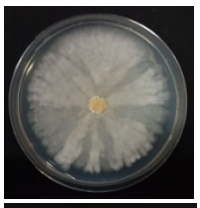
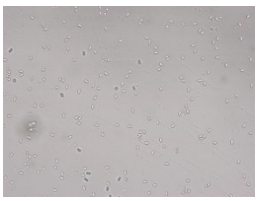
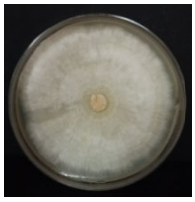
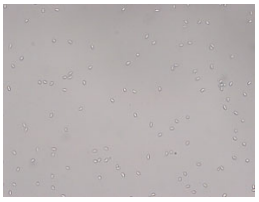
Fig 1 — Effect of different carbon sources on the mycelial growth of *C. rosea* over day

Table 1 — Growth and sporulation of *C.rosea* in different carbon sources

S. No.	Carbon source	Colony morphology	Mycelial growth pattern	Mean radial mycelial growth (cm)	Sporulation	
1.	Arabinose		Aerial, fluffy	4.6^{abc}		++*
2	Carboxy methyl cellulose		Aerial, powdery mass	4.2^{c}		+++*
3	Chitin		Fine, powdery mass	4.5^{abc}		+++*
4	Chitosan		Fine, powdery mass	4.6^{abc}		+++*
5	Dextrose		Aerial, fluffy	4.9^{a}		+++*
6	Mannitol		Aerial, Cottony	4.6^{abc}		++*
7	Pectin		Flat. cottony	4.5^{abc}		+*
8	Sodium acetate		Flat, cottony	4.3^{bc}		++*

(*Contd*.)

Table 1 — Growth and sporulation of *C.rosea* in different carbon sources — (*Contd.*)

S. No.	Carbon source	Colony morphology	Mycelial growth pattern	Mean radial mycelial growth (cm)	Sporulation	
9	Sorbitol		Smooth, adherent	4.6^{abc}		++*
10	Starch		Aerial, fluffy	4.6^{abc}		++*
11	Sucrose		Aerial, fluffy	4.7^{ab}		++*

*+ - poor sporulation (1 – 1.99 × 10^7 spores/mL), ++ - medium sporulation (2 – 2.99 × 10^7 spores/mL), +++ - good sporulation (3 – 3.99 × 10^7 spores/mL)

*Values are mean of three replicates Means in a column followed by same superscript letters are not significantly different according to DMRT test at P ≤ 0.05

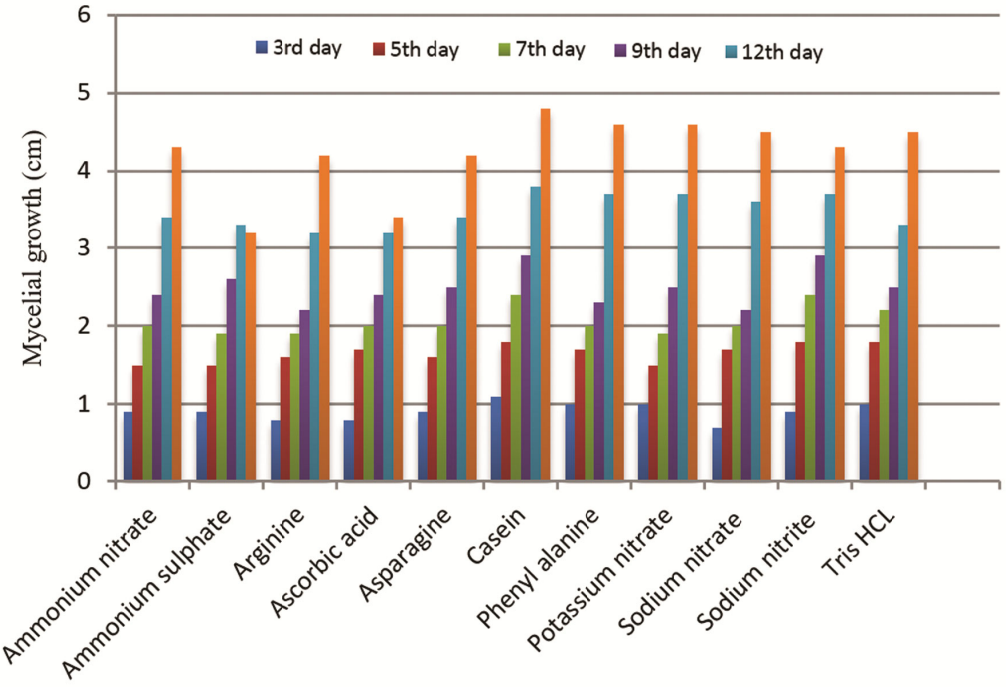
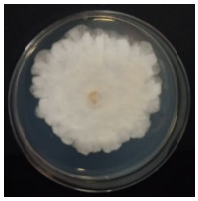
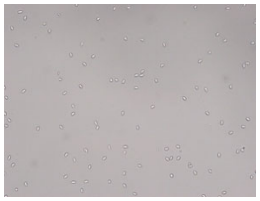
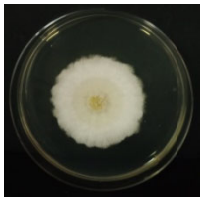
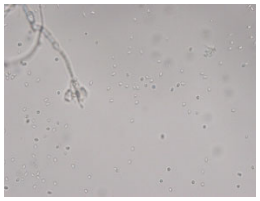
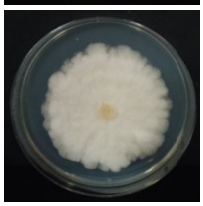
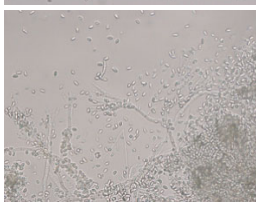
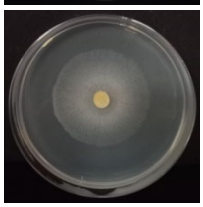
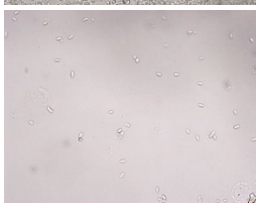
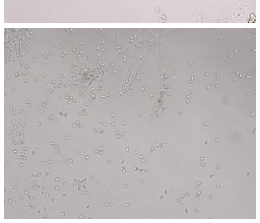
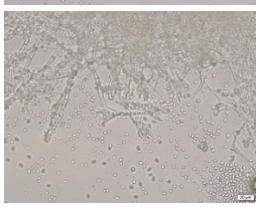
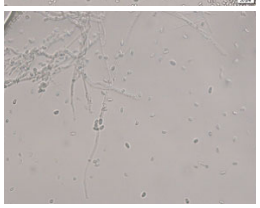


Fig 2 — Effect of different nitrogen sources on the mycelial growth of *C. rosea* over days

C. rosea. Among them, casein was found to be the most suitable nitrogen source which significantly contributed the maximum growth of the mycelium with 4.8 cm (mean radial growth) followed by potassium nitrate, phenylalanine and sodium nitrite with 4.6 cm (mean radial growth) on par (Fig. 2). The

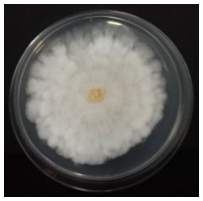
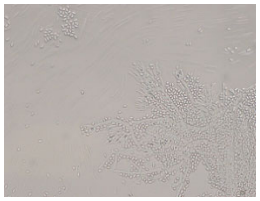
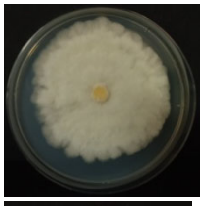
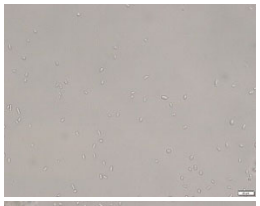
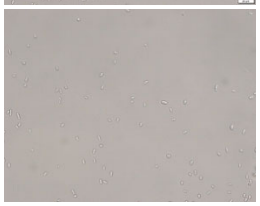
sporulation was also found to be good in all the above nitrogen sources. The least suitable nitrogen source for the growth and sporulation of the isolate was found to be ammonium sulphate with 3.2 cm (mean radial growth) (Table 2). All eleven nitrogen sources were found to be enhancing the growth and sporulation of *C. rosea*, but casein outperformed the other nitrogen sources by contributing significantly. The other best sources were found to be potassium nitrate, phenyl alanine and sodium nitrite was equally

Table 2 — Growth and sporulation of *C. rosea* in different nitrogen sources

S. No.	Nitrogen source	Colony morphology	Mycelial growth pattern	Mean radial mycelial growth (cm)*	Sporulation	
1	Ammonium nitrate		Aerial, fluffy	4.3^{ab}		++*
2	Ammonium sulphate		Flat, fluffy	3.2^{c}		++*
3	Arginine		Aerial, fluffy	4.2^{b}		+++*
4	Ascorbic acid		Smooth, adherent	3.4^{c}		+*
5	Asparagine		Aerial, cottony	4.2^{b}		++*
6	Casein		Aerial, fluffy	4.8^{a}		+++*
7	Phenyl alanine		Aerial, cottony	4.6^{ab}		++*

(*Contd.*)

Table 2 — Growth and sporulation of *C. rosea* in different nitrogen sources — (*Contd.*)

S. No.	Nitrogen source	Colony morphology	Mycelial growth pattern	Mean radial mycelial growth (cm)*	Sporulation	
8	Potassium nitrate		Aerial, fluffy	4.6^{ab}		+++*
9	Sodium nitrate		Flat, fluffy	4.5^{ab}		++*
10	Sodium nitrite		Aerial, fluffy	4.3^{ab}		++*
11	Tris HCL		Flat, semi transparent, filamentous	4.5^{ab}		++*

*+ - poor sporulation (1 – 1.99 × 10^7 spores/mL), ++ - medium sporulation (2 – 2.99 × 10^7 spores/mL), +++ - good sporulation (3 – 3.99 × 10^7 spores/mL)

*Values are mean of three replicates Means in a column followed by same superscript letters are not significantly different according to DMRT test at P ≤ 0.05

improving the growth and sporulation of *C. rosea*. Among the eleven nitrogen sources tested, ammonium sulphate least favours the growth of fungus. Similarly, the maximum mycelial growth of *T. viride* in media containing casein and peptone as nitrogen sources was reported[8] which supports our current investigation. Potassium nitrate was identified as the best source of nitrogen for the growth and sporulation of fungus *Curvularia lunata*[9] which is on par with our studies. In contradictory to our studies, ammonium sulphate was reported as a booster for biomass production of *T. harzianum*[1].

Efficacy and characterization of crude metabolites of *C. rosea* against *FOL*

It is widely known that *Clonostachys* fungus produce a range of secondary metabolites with different biological activity, indicating their use in medicinal and agrochemical applications. In the current study, we have attempted to extract crude metabolites from the efficient isolate TNAU CR 01 which outperformed other isolates in dual assay. After 12 days of incubation, 1000 ppm concentration of crude metabolite from *C. rosea* effectively controlled the mycelial growth of FOL (F1 isolate) by 68% over control followed by 750 ppm with 52 per cent over control (Fig. 3). This clearly shows the activity of antimicrobial compounds in the crude metabolites which should be analyzed using GC-MS.

The GC-MS analysis showed the presence of various antimicrobial and antifungal compounds in secondary metabolites of *C. rosea* (Table S1). The compounds were identified and confirmed through NIST library 2005. The most important compounds identified were Itraconazole, Mesoporphyrin IX, Dasycarpidan-1-methanol, acetate (ester), 1, 25-Dihydroxyvitamin D3, TMS derivative, 9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol which has antifungal and anti microbial properties. The compound

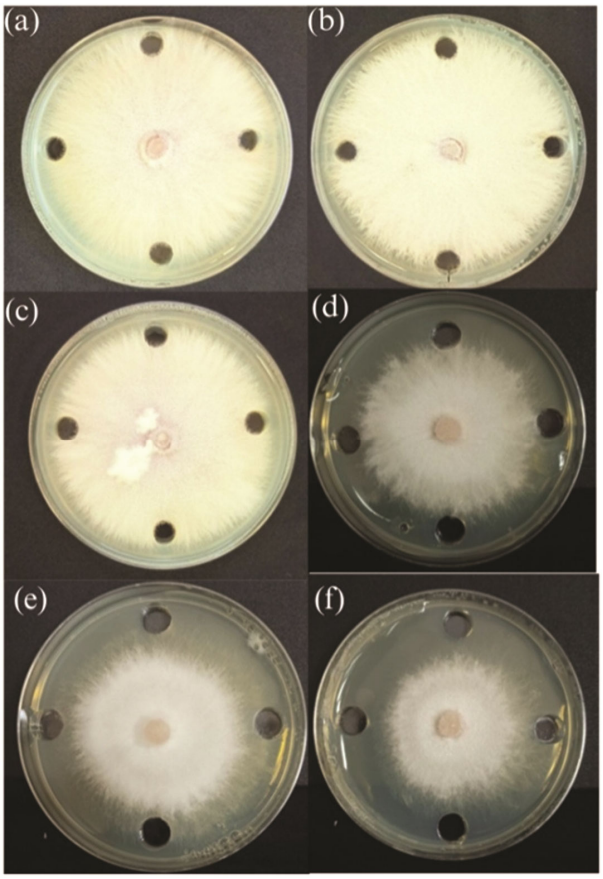


Fig 3 — Efficacy of crude metabolites of *C. rosea* over FOL (a) methanol control, (b) sterile water control, (c) 250 ppm, (d) 500 ppm, (e) 750 ppm and (f) 1000 ppm

Itraconazole was found to be present in *C. rosea* isolate (TNAU CR 01) which has the anti-fungal and anti-protozoal properties. The biological effect of the compound Itraconazole and its anti fungal activities against fibroblasts[10] supports our current findings. Mesoporphyrin IX compound was also identified which was reported to have antimicrobial properties against *Enterococcus faecalis*[11]. Other important compounds viz., Dasycarpidan-1-methanol, acetate (ester), 1,2,5-Dihydroxyvitamin D3, TMS derivative, 9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol were also found to have antifungal and antimicrobial properties against wide range of pathogens[12,13,14] which supports our present work.

Conclusion

In conclusion, we recommend dextrose and casein as an effective carbon and nitrogen sources, respectively, for the efficient growth and sporulation of *C. rosea* and can be used for biomass production when it comes to commercialization of the antagonistic fungus to combat economically important diseases like *Fusarium oxysporum* f.sp. *lycopersici*. The antimicrobial and antifungal properties of the fungus *C. rosea* have been identified in the present study through GC-MS analysis which further supports their commercialization as an efficient biocontrol agent.

Acknowledgment

The authors acknowledge the Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu for the support of entire research work.

Supplementary Information

Supplementary information is available on the website http://nopr.niscpr.res.in/handle/123456789/55.

References

1 Rai D & Tewari A K, *Int L Agric Biotechnol Sust*, 8 (2016) 67.
2 Safavi S A, Shah F A, Pakdel A K, Reza R G, Bandani A R & Butt T M, *FEMS Microbiol Lett*, 270 (2007) 116.
3 Mustafa U & Kaur G, *Folia Microbiol*, 54 (2009) 499.
4 Han P, Zhang X, Xu D, Zhang B, Lai D & Zhou L, *J Fungi*, 6 (2020) 229.
5 Sun Z B, Sun M H, Zhou M & Li S D, *AMB Express*, 7 (2017) 1.
6 Mehta J, Jakhetia M, Choudhary S, Mirza J, Sharma D, Khatri P, Gupta P, Meenu & Nair M, *European J Exp Biol*, 2 (2012) 2061.
7 Moura R D, de Castro L A M, Culik M P, Fernandes A A R, Fernandes P M B & Ventura J A, *J Microbiol Methods*, 173 (2020) 105915.
8 Juwon A D & Emmanuel O F, *Biotechnol Res Int*, 2012 (2012) 904763.
9 Lal M, Ali M, Kumar S, Singh V & Khan A, *Bioscan*, 9 (2014) 1197.
10 Abe S, Ochi H, Takahashi Y, Ishijima S A, Osumi M & Yamaguchi H, *J Infect Chemother*, 6 (2000) 35.
11 Salageanu L, Muntean D, Licker M, Lascu A, Anghel D & Fagadar-Cosma E, *Farmacia*, 68 (2020) 288.
12 Ghaidaa J M, Mohammad J Al-J & Imad H H, *Int J Pharmacogn Phytochem Res*, 8 (2016) 480.
13 Shareef H K, Muhammed H J, Hussein H M & Hameed I H, *Orient J Chem*, 32 (2016) 817.
14 Wang T T, Nestel F P, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan J W, Mader S & White J H, *J Immunol*, 173 (2004) 2909.