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Standardization of Various Parameters for Mycoherbicidal metabolites production from Fusarium sp. FGCCW#16 for Parthenium hysterophorus Management

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ABSTRACT

Parthenium hysterophorus weeds are one of the major problems in almost all types of agricultural lands, forest lands, pastures, farmlands and disturbed land including roadsides. It is also responsible for health problems in human and animals besides loss to agriculture and ecosystems. It causes allergic respiratory problems, contact dermatitis and mutagenicity. The main strategy of *Parthenium* control is the use of chemical herbicides. The increasing side effects of chemical herbicide and growing resistance against them in weeds have attracted the attention of researchers to search for some novel herbicidal compounds from natural sources. A significant barrier in the mycoherbicide metabolites production is the development of an economically viable fermentation process. The production of these compounds is largely affected by certain parameters like pH, temperature, incubation days and media constituents etc. Adjustment of these parameters up to optimum level leads to maximum production of mycoherbicidal compounds. Therefore, the purpose of this study was to standardize physiochemical conditions (Nutrient media, pH, Temperature, and Incubation periods) for maximum production of mycoherbicidal metabolites from phytopathogenic fungi *Fusarium* sp. FGCCW#16 isolated from Parthenium hysterophorus weed. *Fusarium* sp FGCCW#16 grew well and produced optimum mycoherbicidal metabolites in Richard's broth medium at 25 to 28°C on the 21 studay of the incubation. The optimal growth, however, was obtained at pH 6. The results of this investigation indicated that cultural conditions like culture media as well as physical factors such as temperature, pH and incubation period greatly affected the growth and production of mycoherbicidal metabolites by *Fusarium* sp FGCCW#16.

Introduction

The weed *Parthenium hysterophorus* are serious problems not only for agricultural, disturbs cultivated areas, roadside vegetation, gardens and forestry fields, but also responsible for several

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major problems to human and animal health around the world including India. Synthetic chemical herbicides have been the mainstay for *Parthenium* control practices. Weed resistance to herbicides, elimination of some older herbicides, the high cost for development and registration of new chemical herbicides, lack of herbicides registered for small markets are major factors (Charudattan, 2001). So today organic agriculture need tools to manage weeds and reduce their reliance on synthetic herbicides (Cordeau et al. 2016). These facts lead to a growing search for new herbicides with safer toxicological and environmental profiles as well as with new modes of action (Dayan and Duke, 2014). The search for novel mycoherbicidal activities is of great importance for agriculture, since most existing herbicides are either banned or inefficient due to resistant weeds, and the flow of novel herbicides identified by classical chemical screening is dramatically reduced. Fungi are capable of producing phytotoxic metabolites. These compounds are present in the culture medium or substrate where fungi are growing, originating from primary or secondary metabolism.The secondary metabolites produced by filamentous fungi which demonstrate toxic properties in plants are called phytotoxins. It is observed that some fungi can produce phytotoxins thus can be used for weed control. Fungi, such as *Alternaria, Fusarium, Colletotrichum* and *Phoma* can produce phytotoxin (Singh, 2007). These natural compounds remain under-explored, mainly because they are only produced during interaction with host plants (infection-specific expression patterns). Various state-of-the-art techniques used to extract and purify the metabolites produced by these fungal strains using large-scale cultures. A number of bioactive secondary metabolites produced by fungi out of which herbicidal metabolites are the most important ones (Strobel and Daisy, 2003; Keller et al 2005). But the production of these metabolites is largely influenced by the nutritional as well as environmental factors associated with the fungi. The nutritional factors include carbon source, nitrogen source etc. and environmental factors include pH, temperature, incubation period (Thakur et al. 2009). The requirement of above conditions varies from fungus to fungus depending upon the genera to which they belong (Stanbury, 1995). Optimization of such factors is necessary for obtaining highest yield of secondary metabolites. *Fusarium sp* FGCCW#16 is a phytopathogenic fungi having herbicidal potential which was isolated from infected leaf of *Parthenium hysterophorus* weed in a previous study (Singh, 2007). Phytopathogenic fungi are a tremendous source of bioactive secondary metabolites which are partially unexplored. Optimization of various physiological factors for production of bioactive compounds by fungi has been done by many scientists and critically reviewed in many publications (Merlin et al. 2013; Mathan et al. 2013; Ismaiel et al. 2010; Jyotsnamayee and Gupta, 2010; Boonyapranai et al. 2008; Gunasekaran et al. 2008). Therefore, the present research paper carried out to determine the optimum conditions suitable for maximum production of herbicidal metabolites by *Fusarium* sp FGCCW#16.

Materials and Methods

Standardization of Various Parameters for Maximum Mycoherbicidal Metabolites Production

Different physiochemical parameters were studied for maximum production of mycoherbicidal metabolites by selected strain *Fusarium* sp. FGCCW#16. These parameters are presented below.

Standardization of Nutrient Media

The selected strain FGCCW#16 was grown in various semi-synthetic and synthetic media to select suitable media for maximum herbicidal metabolite production during the course ofthe present study. During the fermentation process, cell growth (Biomass) and the yield of herbicidal metabolites by the strain were recorded. The medium in which the strain exhibits optimum levels of herbicidal metabolites in terms of herbicidal and biomass production was selected for further studies. All the chemicals used in the preparation of media in the present investigation belong to either of these standard companies *viz*., CDH, Hi-Media, Merck, Qualigens, SRL, and S.D. Fine. Compositions of these media are presented below.

Semi Synthetic Media

Miller's Broth (Glucose: 0.075 gm; Peptone: 0.075 gm; Yeast Extract: 0.075 gm; D/W: 150 ml), Sabouraud's Dextrose Broth (Glucose: 0.6 gm; Peptone;1.5 gm; D/W: 150 ml), Fresh Potato Dextrose Broth (Potato Extract: 200 ml; Glucose: 20 gm; D/W: 1000 ml), Maltose Peptone Broth (Maltose: 4 gm; Peptone: 20 gm; D/W: 1000 ml), Martin's Broth (Dextrose: 1.5 gm; Peptone: 0.75 gm; KH_2PO_4: 0.15 gm; $MgSO_4$. 7 H_2O: 0.075 gm; D/W: 150 ml), Starch Nutrient Broth (Starch: 3 gm; Peptone: 0.75 gm; Beef Extract: 0.45 gm; D/W: 1000 ml).

Synthetic Media

Czapek DoxBroth ($NaNO_3$: 2 gm; KH_2PO_4: 1 gm; $MgSO_4$. 7 H_2O: 0.5 gm; KCl: 0.5 gm; $FeSO_4$. 7 H_2O:Trace (0.02)gm; Sucrose: 30 gm; DW: 1000 ml), Cohn's Medium (KH_2PO_4: 5 gm; $MgSO_4$.7 H_2O: 0. 5 gm; Ammonium tartarate: 10gm; KCl: 0 5. gm; DW: 1000 ml), Asthana& Hawker's media (KNO_3: 3.5 gm; KH_2PO_4: 1 gm; $MgSO_4$. 7 H_2O: 0.5 gm; Glucose: 5 gm; DW: 1000 ml), Coons Broth($NaNO_3$: 2 gm; KH_2PO_4: 1 gm; $MgSO_4$. 7 H_2O: 0.5 gm; DW: 1000 ml), Richards Broth (KNO_3: 10 gm; KH_2PO_4: 5 gm; $MgSO_4$. 7 H_2O: 2.5 gm; $FeCl_3$: Trace (0.02)gm; Sucrose: 35 gm; DW: 1000 ml), Pferrer's Medium (Ammonium nitrate: 10gm; KH_2PO_4: 5 gm; $MgSO_4$. 7 H_2O: 2. 5gm; Sucrose: 50 gm; $FeSO_4$. 7 H_2O: Trace (0.02)gm; DW: 1000 ml), Fermi's Medium ($MgSO_4$. 7 H_2O: 0. 2 gm; KH_2PO_4: 1 gm; Ammonium sulphate: 10gm; glycerine: 45ml; DW: 1000 ml).The biomass and

mycoherbicidal metabolite production were estimated by comparing the biomass and quantified clear crude metabolites and phytotoxicity testing with *Parthenium* Shoot cut Bioassay.

Determination of Incubation period

Mycoherbicidal metabolite productions by the selected strain were observed at different incubation periods *viz*., 7, 14, 21 and 28 days. The period of incubation which yielded maximum metabolite was considered as optimum period. The biomass and mycoherbicidal metabolite production were estimated by comparing the biomass and quantified clear crude metabolites and phytotoxicity testing with *Parthenium* Shoot cut Bioassay.

Effect of pH

Effect of initial pH on growth and herbicidal metabolite production of the strain was determined by adjusting the pH of production medium ranging from 3-12. The pH of the basal medium was adjusted by addition of 1 NHCl or NaOH before autoclaving and Systronic pH meter was used for pH measurements. The pH exhibiting highest herbicidal metabolite production was selected as optimum pH for further studies. The biomass and mycoherbicidal metabolite production were estimated by comparing the biomass and quantified clear crude metabolites and phytotoxicity testing with *Parthenium* Shoot cut Bioassay.

Effect of Temperature:

Optimum temperature for herbicidal metabolite yield was determined by incubating the production medium at different temperatures varying from 0^0C, 5^0C, 10^0C 15^0C, 20^0C, 25^0C, 28^0C, 30^0C, 35^0C and 40^0C maintaining all other conditions at optimum levels. Temperature showing highest herbicidal metabolite production was taken as the optimum. The biomass and mycoherbicidal metabolite production were estimated by comparing the biomass and quantified

Results and Discussion

Standardization of various parameters for maximum mycoherbicidal metabolites production

Environmental factors are typically highly variable for the mycoherbicidal metabolites production. Various factors comprising the internal or external environment may affect either the amount of secondary metabolites or their activity (Liu and Li, 2004; Liu and Chen, 2002). Being a biological form it is indispensable for fungi to acquire the materials essential for the synthesis of cellular constituents and functional purpose. The principle elements requiredby fungi are carbon, nitrogen, phosphorus, sulphate ions, potassium, magnesium, iron, zinc and manganese. To optimize

growth, toxin production, carbon, nitrogen and other nutrientslevels requires precise balancing (Gbolagade, 2006; Churchill, 1982). Physiochemical requirements of fungi are known to vary significantly within not only in genera but also strains of isolates (Stanbury et al. 1995; Lilly and Barnett, 1951). There are no generalized conditions which are applicable to all the fungi. Therefore, the present investigation was carried out to determine the optimum physiochemical conditions suitable for maximum production of mycoherbicidal metabolites by *Fusarium* sp FGCCW#16.

Standardization of Nutrient Media

Selection of a suitable medium for mycoherbicidal production is an important step. Such amedium was a prerequisite for further studies. The findings of this study showed that different culture media influenced the mycoherbicidal metabolite production of *Fusarium* spp FGCCW#16. Data recorded in the Table 1 shows that Richard's broth produced maximum crude mycoherbicidal metabolite production by *Fusarium* sp FGCC#16 after 21 days of incubation at 28^0C. It was followed by potato dextrose, maltose dextrose broth, Asthana and Hawker's media, Coons Broth and Czapeck broth.

Table 1. Study different nutrient media effects on metabolites production and phytotoxicity study by Parthenium Shoot Cut Bioassay.

S.N	Media Name	Crude Metabolite (Grams/Litre)	Phytotoxicity			
Semi Synthetic Media			24 hrs	48 hrs	72 hrs	96 hrs
1.	FPDB	8	1*	2	3	4
2	Maltose Peptone Broth	9.5	0	0	1	1
3	Starch Nutrient Broth	16	1	2	2	2
4	Martin's Broth	12	2	3	3	4
5	Miler's Broth	11.5	1	2	2	2
6	Sabouraud's Dextrose Broth	8.00	2	3	3	4
		Synthetic Media				
7	Cohn's Medium	9.00	1	2	2	2
8	CzapekDox Broth	9.5	2	3	3	4
9	Richards Broth	19.0	2	3	4	5
10	Coons Broth	12.00	1	2	3	3
11	Asthana & Hawker's Media	10.00	1	2	2	3
12	Fermi's Medium	9.5	0	0	0	0
13	Pferrer's Medium	9.5	0	0	0	0

Incubation Days: 21 Days; Incubation Temperature: 28^0C; Initial pH: 6

*Phytotoxic Damage Rating: 0-No Effect; 1-Curling of leaf; 2-Yellowing of leaf; 3-Browing and Drooping of Leaf; 4-Complete necrosis and Chlorosis; 5-Complete Death.

Boonyapranai et al. (2008) reported maximum production of secondary metabolite from *Fusarium verticillioides* in potato dextrose broth medium. Oatmeal agar followed by Richard's agar, Czapek dox agar and Potato dextrose agar resulted inmaximum production of secondary metabolites compounds from *Fusarium oxysporum* by Chittem and Kulkarni 2008. Maximum phytotoxic compound production was observed from *Fusarium oxysporum* in Potato dextrose broth medium (Lee et al. 2008). Potato sucrose broth was found to be the most suitable media for the production of herbicidal compounds by *Fusarium oxysporum* (Parmar et al. 2010). Similar observations have also been recorded by many other workers (Bhattacharyya and Jha, 2011; Lee et al. 2008). Richard'sbroth has also been reported as best medium for metabolite production by different fungi (Lakpale et al. 1996; Gaur and Agnihotri, 1982). Shoot cut bioassay was also performed with different media fungal extracts and the results are represented in Table 1. Maximum phytotoxic damage to *Parthenium* shoots occurred with Richard' broth. Maximum phytotoxic damage was recorded with fermented media containing Richard'sbroth followed by potato dextrose, maltose dextrose broth, Asthana and Hawker's media, Coons Broth and Czapeck broth. On the basis of overall performance and synthetic in nature, Richard's broth was selected as best medium for further experimentation.

Determination of Incubation period

Time duration required for growth and metabolite production by different fungi varies significantly (Alberts et al. 1990).Thus, proper determination of specific incubation requirement is of highimportance for maximum harvesting of the metabolite. It is evident from the data recorded in Table 2 that production of mycoherbicidal metabolite varied significantly with incubation days. Maximum mycoherbicidal metabolite production was recorded in 21 day's old fermented broth. It was followed by 14 days and 28 days of incubation. Tanaka (1996) has also reported variation in mycoherbicidal metabolite production with incubation period. The 9 days incubation period was found tobe optimum for growth and secondary metabolite production in *Fusarium solani* by Merlin et al (2013). Metabolites produced at 21 days incubation produced maximum damage within 96 hrs. Phytotoxic effect by the toxin was minimum after 12 hpt and maximum after 96 hpt. Similar trend was also recorded by earlier workers (Singh, 2007; Pandey et al. 2004). Thus incubation 21 days was optimum for phytotoxin production where highest quantities of toxic metabolite production was observed. A sharp difference in phytotoxicity with increasing incubation period may be credited to different phases of growth of the growing fungus. Metabolites are also required for the growth of fungus itself and are normally synthesized during initial phase whereas most of the toxicants are produced during stationary phase i.e. idiophase of the mould. Maximum

mycoherbicidal activity in CFCF of 7 days old culture broth of *Fusarium roseum* was reported by Ismaiel et al. (2010). Parmar et al. (2011) reported highest yield of fusaric acid and also the herbicidal activity after 10th day of incubation. Thus, in further experiment 21 days old culture filtrate was used.

Table 2. Study of effect of different incubation periods on metabolites production and phytotoxicity study by *Parthenium* Shoot Cut Bioassay.

S.N	Incubation days	Crude metabolites (Grams/Litre)	Phytotoxicity			
			24hrs	48hrs	72hrs	96hrs
1	7	8	1*	1	2	2
2	14	9.5	1	2	3	3
3	21	18	2	3	4	5
4	28	10	1	2	3	3

Medium: Richard's Medium, Incubation Temp: 28^{0}C; Initial pH: 6.
*Phytotoxic Damage Rating: 0-No Effect; 1-Curling of leaf; 2-Yellowing of leaf; 3-Browing and Drooping of Leaf; 4-Complete Necrosis and Chlorosis; 5-Complete Death.

Effect of pH

The pH of a culture medium is usually not constant throughout fermentation and the changesthat occur are highly dependent on composition of the medium. The optimum pH for growth rate may be entirely different from optimum pH of product formation (Merlin et al. 2013). In order to determine the optimum pH for maximum mycoherbicidal metabolite production the test strain was grown in basal medium with different hydrogen ion concentrations. Data presented in Table 3 clearly reveals that the test strain could grow within a wide range of pH. Maximum crude mycoherbicidal compound production was obtained at pH level 4 followed by pH 5 and 6. Metabolites produced at pH 4 produced maximum phytotoxic damage within 12 hrs, which enhanced till 48 hrs. Phytotoxic effect by metabolites was minimum after 12 hrs and maximum after 96 hrs. Similar trend was also recorded by earlier workers (Singh, 2007; Pandey et al. 2004). Thus pH 4 was optimum for phytotoxin production where highest quantities of toxic metabolite production were observed. Similar observations have alsobeen recorded by many earlier workers (Mathan et al. 2013; Boonyapranaiet al. 2008). The optimum pH for the growth and secondary metabolite production of *Fusarium solani* was found to be 6.0 by Merlin et al. (2013). On the basis of present investigation pH 4.0 was selected for further investigation.

Table 3. Study of effect of different pH on metabolites production and phytotoxicity damage observation by *Parthenium* Shoot Cut Bioassay.

SN	pH level	Crude metabolites (Grams/Litre)	Phytotoxicity			
			24hr	48hr	72hr	96 hrs
1	3	10 gms	1*	2	3	3
2	4	16 gms	2	3	4	5
3	5	12 gms	2	3	4	5
4	6	11 gms	2	3	4	4
5	7	7 gms	1	1	2	2
6	8	6 gms	1	1	1	1
7	9	6 gms	1	1	1	1
8	10	5 gms	1	1	1	1
9	11	3 gms	0	1	1	1
10	12	3 gms	0	1	1	1

Media: Richard's Media; Incubation Days: 21 Days; Incubation Temp: 28^0C.
*Phytotoxic Damage Rating : 0-No Effect; 1-Curling of leaf; 2-Yellowing of leaf; 3-Browing and Drooping of Leaf; 4-Complete necrosis and Chlorosis; 5-Complete Death.

Temperature

Incubation temperature is known to influence directly the overall growth and development of any organism and it affects the physiology and subsequently the synthesis of various metabolites (Pandey et al. 2005). Optimal temperatures are required for vegetative growth and metabolites production. The incubation temperature is one of the significant parameter in determining the overall growth and development of any organism. Effect of different incubation temperature on growth and phytotoxin production *Fusarium* sp FGCCW#16 was studied by shoot cut bioassay on *Parthenium* shoots and is shown in Table 4. Maximum production was achieved at 25°C followed by 28°C and 30°C. No significant phytotoxin was reported at lower and higher incubation temperatures. Phytotoxin produced at 25, 28 and 30°C produced remarkable phytotoxicity. Phytotoxic damage on *Parthenium* leaves on treatment with crude toxin produced at various incubation temperatures is given in Table 4. Phytotoxin produced at 25°C exhibited maximum damage to the leaves followed by 28°C and 30°C. Maximum mycoherbicidal compound production was obtained at 25°C and beyond this it gradually declined. Much lower as well as higher temperature failed to support any growth or herbicidal compound production. Significant variation in phytotoxin production by other fungi at various temperatures has also been observed (Pandey et al. 2004). The temperature 25°C was reported as the optimum temperature for maximum bioactive

metabolite production from *Aspergillus terreus* KC588297 (Mathan, 2013). Similarly, the best temperature for the growth and metabolite production of *Fusarium solani* was found to be 25±2^{0}C by Merlin et al. (2013). In the present investigation, mycoherbicidal compound production occurred at 25°C. Gunasekaran and Poorniammal (2008) reported highest secondary metabolite production at a temperature of 30°C. According to Parmar et al. (2010) highest fusaric acid production by *Fusarium oxysporum* was obtained at 25°C.

Table 4. Study of effect of different temp on metabolites production and phytotoxicity damage observation by *Parthenium* Shoot Cut Bioassay.

SN	Incubation Temp (^{0}C)	Crude metabolites (Grams/Litre)	Phytotoxicity Study				
			12 hrs	24hrs	48hrs	72 hrs	96 hrs
1	0	2	0*	0	0	1	1
2	5	3	0	0	0	1	1
3	10	3	0	0	0	1	1
4	15	11	0	1	1	1	1
5	20	14	1	2	3	3	4
6	25	16	2	3	4	5	5
7	28	12	2	3	3	4	5
8	30	11	1	2	3	4	4
9	35	6	1	1	1	1	1
10	40	6	1	1	1	1	1

Media: Richard's Media; Incubation Days: 21 Days; Incubation pH: 4.
*Phytotoxic Damage Rating: 0-No Effect; 1-Curling of leaf; 2-Yellowing of leaf; 3-Browing and Drooping of Leaf; 4-Complete necrosis and Chlorosis; 5-Complete Death.

Conclusion

With the development of sustainable agriculture and consciousness of human environmental protection, government and enterprises will pay more attention to the study and exploitation of microbial herbicide, due to their potential benefits for the environment. Thus, this paper brings concept of the biological control as a promising alternative for weed control and shows the need for scientific research on the agents and the techniques that should be mastered for mycoherbicide development and production. Besides the potential of mycoherbicide in modern agriculture, however, few have achieved long-term commercial success. Thirteen bioherbicides derived from microorganisms or natural molecules are currently available on the market: nine are based on fungi, three on bacteria and one based on active substance obtained from natural plant extract (Cordeau et al. 2016). Therefore, the demand for bioherbicides has increased, but on the other

hand, more research is needed to discover new biological control agents to improve the existing agents' action and also to optimize production processes. Fungal eco-friendly agribiologicals or fungal phytotoxins are plant protection molecules gaining momentum for agrichemical research nowadays. My work has actively been involved with isolation of phytopathogenic fungi, extraction of phytotoxins, determination of their herbicidal potential and mass production and formulation of herbicidal metabolites. We have isolated phytotoxins from various fungi for management of some prominent weeds of Central India. Thus in my present study, optimization of culture conditions for mycoherbicidal compound production by *Fusarium* sp FGCC#16 was achieved under optimal culture conditions by using shake flask fermentation. The optimization of physiochemical properties of mycoherbicidal compound produced by *Fusarium* sp FGCC#16 and its herbicidal activity against weeds *Parthenium*. It demonstrates the feasibility of commercial production of this mycoherbicidal compound as potential herbicidal compound after further investigations. These findings will assist in formulating the culture medium and other factors which are suitable for producing the herbicidal bioactive compounds from *Fusarium* sp FGCCW#16.

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Conflict of Interest

Authors declare no conflict of interest.

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