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Fungal Endophytes from Maize (*Zea mays* L.): Isolation, Identification and Screening against Maize Stem Borer, *Chilo partellus* (Swinhoe)

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In this study, nine maize varieties (NAH-1137, CM-500, NAC-6004, Nithyashree, Seed tech-2324, Bio-9637, Bio-9681, DHM117 and COH(M)10) plants were grown at NBAIR farm, Bangalore, India, from January 2015 to April 2015. A total of 17 fungal endophytes isolated from leaf, stem and root fragments collected about thirty, sixty and ninety days after seeds sown. The fungi were identified as *Acremonium zeae, Cladosporium oxysporum, Colletotrichum boninense, Colletotrichum gloeosporioides, Coprinopsis cinerea, Curvularia lunata, Epicoccum sorghinum, Fusarium fujikuroi, Gibberella moniliformis, Nemania sp., Penicillium sp., Rigidoporus vinctus, Sarocladium zeae* and *Scopulariopsis gracilis.* Difference was found in the number of fungi isolated from leaves, stems and roots irrespective of maize varieties. Presence of fungal endophytes were more in leafs than stems and roots of maize. Irrespective of maize tissues, more fungi were isolated about sixty days after seeds sown. The isolate *Furarium fujikuroi* showed higher colonization with 34.02%, followed by the genera *Penicillium* with 17.01% colonization. Furthermore, fungal endophytes were evaluated for their pathogenicity against *C. partellus*, among the endophytes tested *Cladosporium oxysporum* and *Rigidoporus vinctus* isolates showed 6.67 and 10% mortality respectively.

Keywords: Endophyte, Maize, *Chilo partellus*, Biocontrol.

Maize is one of the important cereal crop cultivated in different parts of the world. It is widely used for animal and human consumption. Maize is the third major crop in India, it has great importance for grain and fodder purpose. Maize is also used for production of oil, syrup, alcohol, acetic, lactic acid, glucose, gum, starches for edible and laundry purpose, adhesives, methanol, corn meal and flakes. Around 140 insect pests causes damage in maize, among them ten causes severe yield loss. *Chilo partellus* (Swinhoe) is one of the most important pest for causing yield loss in maize, it mainly attack the crop during *kharif* season. Control of borer pest is highly difficult by chemical insecticides because of borer cryptic life cycle and chemical insecticides are not safe for both environment and non target organisms. In this manner, need of safe and promising biocontrol agent for control of insect pests.

Endophytic microorganisms are group of organisms they are colonizing inside the plant tissues without causing apparent symptoms. Endophytes also survive in intercellular space or within cells of host plant causing no apparent damage (Saikkonen *et al*., 1998). Under stress conditions, endophytes cause disease symptoms of host plant (Fu-kang Gao *et al.*, 2010). Fungal endophytes providing protection to the host plant

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against herbivorous insects (Clement *et al.*, 1994), plant pathogens (Dingle and McGee, 2003) and adverse environmental conditions such as heat, stress and prolonged periods of drought (Andrea Porras-Alfaro and Paul Bayman, 2011). These fungal endophytes were exploited as biocontrol agent of plant pathogen and insect pest.

This present investigation aimed primarily to isolation and identification of fungal endophytes. At the same time, evaluate the pathogenicity of fungal endophytes against maize stem borer *C. partellus*.

MATERIALS AND METHODS

Sample collection

Nine maize varieties like, NAH-1137, CM-500, NAC-6004, Nithyashree (Seeds were obtained from College of Agriculture, V.C. Farm, Mandya) Seed tech-2324, Bio-9637, Bio-9681, DHM117 and COH (M)10 (Seeds were obtained from Maize Research Centre, Hyderabad) plants grown at NBAIR farm, Bangalore, India, during January 2015 to April 2015. Leaf, stem and root samples of maize were collected about thirty, sixty and ninety days after seeds sown. Each of the sampling periods, a total of 9 healthy plants were randomly collected from each of the nine varieties. After the collection, plant parts were washed with tap water and processed immediately for isolation of fungal endophytes.

Isolation of fungal endophytes

As per the procedure of Nur Amin (2013) endophytic fungi were isolated from the healthy maize plants. All the leaf, stem and root samples of maize collected from the field were washed in tap water then surface sterilized by immersion in 70% (v/v) ethanol for 1 minute, followed by 1% (v/v) sodium hypochlorite for 5 minutes and 30 seconds in 70% (v/v) ethanol and then washed three times in sterilized distilled water for 1 minute. After surface sterilization, the samples were cut into 5 mm pieces and transferred aseptically into petri dishes containing potato dextrose agar (PDA) (Potato 200g, dextrose 20g, agar 15g per 1 litter distilled water.) with chloramphenicol (100 mg/ml). Aliquots from final washed water were also plated on the PDA plates to check the effectiveness of surface sterilization. A total of 243 bits were plated from each of the nine varieties. Plates were incubated at room temperature up to 20 days. Plates were examined after 20 days of incubation and any endophytic fungi emerged from plant tissues was isolated and purified. These isolates were maintained at 4^{o}C on PDA slants for further identification studies. Percent colonization of fungal endophytes was calculated according to Carroll and Carroll, 1978.

Characterization of fungal endophytes

DNA isolation and PCR studies

Total genomic DNA was isolated from mycelial mat by using plant DNA isolation kit (CTAB method). For identification, ITS1 (5'-TCCGTAGGTGAACCTGCGG), ITS4 (5'-TCCTCCGCTTATTGATATGC), AB28 (5'-ATATGCTTAAGTTCAGCGGGT) and TW81 (5'-GTTTCCGTAGGTGAACCTGC) primers were used to amplify the nuclear ribosomal internal transcribed spacer region (ITS) by using PCR. Final volume of PCR mixture (50µl) consisting of 50ng of fungal genomic DNA , 50 pmol each of Primers, 1.25mM for each of dATP, dGTP, dCTP, dTTP, 2.5 units of Taq DNA polymerase , 2µl of polymerase buffer, 2.5 Mm $MgCl_2$ and sterile water to makeup 50µl of total volume. The PCR program comprised initial denaturation at 95^{o}C for 2 min, followed by 36 cycles at 94^{o}C for 30 seconds, 60.6^{o}C for 30 seconds, 72^{o}C for one min and final step at 72^{o}C for 10 min for extension. Quantarus Thermal cycler was used to carry out the cycles (Ramanujam *et al*., 2011). For species identification, sequences were blasted by using BLAST search for the ITS sequence of rDNA available in the NCBI GenBank database.

Screening of fungal endophytes against *C. partellus*

The fungal endophytes isolated from healthy maize tissues were screened in vitro to evaluate the pathogenicity against maize stem borer, *C. partellus*.

Preparation of conidial suspension

Conidial suspension of each fungal isolate was prepared by scraping conidia from 15-day-old culture from Petri plate into sterile distilled water with 0.002% Tween 80. Suspension was filtrated through three layers of muslin cloth to get hyphal-free conidial suspension. The conidial concentration in the suspension was adjusted to $1x10^8$ conidia/ml using Neubauer' s improved haemocytometer (Quesada-Moraga *et al*., 2006).

Bioassays

The experiment was conducted according to the protocol of Safavi *et al*., (2010). Conidial suspension (1x10^{8}conidia/ml) of each fungal isolate was prepared for bioassay studies. Field collected *C. partellus* larvae were reared in the laboratory according to the protocol Chandish R Ballal *et al*., 1995. Fifteen numbers of second instar *C. partellus* larvae were dipped in one ml of conidial suspension of each fungal endophyte for 30 seconds. Control larvae were treated with sterile distilled water with 0.1% Tween 80. All treatments were replicated three times. Treated larvae were transferred into a sterile plastic container and fed on maize leaf bits. Larval mortality was recorded up to 10 days at 24h intervals. The dead larvae were transferred to sterile petri dishes lined with moist filter paper to facilitate mycosis. Abbott's formula (Abbott, 1925) was used to calculate the per cent mortality of *C. partellus*.

RESULTS

Isolation and identification of fugal endophytes

A total of 17 fungal endophytes were isolated from healthy maize tissues. Among them, 11 fungi were isolated from leaf, 4 fungi from stem and 2 fungi from root tissues of the maize plants. These fungal endophytes were identified as *Acremonium zeae, Cladosporium oxysporum, Colletotrichum boninense, Colletotrichum gloeosporioides, Coprinopsis cinerea, Curvularia lunata, Epicoccum sorghinum, Fusarium fujikuroi, Gibberella moniliformis, Nemania sp., Penicillium sp., Rigidoporus vinctus, Sarocladium zeae* and *Scopulariopsis gracilis*. The isolate *F. fujikuroi* showed highest colonization with 34.02%, followed by the genera *Penicillium* with 20.01% colonization. *E. sorghinum* (18.63), *S. zeae* (18.63) and *Nemania sp*. (8.1) were most frequently isolated fungi from maize. Irrespective of maize varieties, among the fungal endophytes isolated, 6 isolates were isolated when the plants were 30 days old, 9 isolates from 60 days old plants and 2 isolates from 90 days old plants (Table. 1).

Table 1. List of fungal endophytes isolated from maize

Variety	Leaf						Stem						Root					
	30 days		60 days		90 days		30 days		60 days		90 days		30 days		60 days		90 days	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Bio-9681	*Penicillium sp.*	20.01	-	-	-	-	-	-	*E. sorghinum*	10.53	-	-	-	-	-	-	-	-
	A. zeae	6.48	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
Seed Tech 2324	-	-	*C. lunata*	2.43	*C. oxysporum*	8.91	-	-	-	-	-	-	-	-	-	-	-	-
COH(M)10	-	-	*Nemania sp.*	5.67	-	-	*C. boninense*	9.72	-	-	-	-	*S. gracilis*	7.29	-	-	-	-
NAH-1137	*C. gloeosporioides*	8.1	-	-	-	-	-	-	*Nemania sp.*	2.43	-	-	-	-	-	-	-	-
DHM117	-	-	-	-	*G. moniliformis*	3.24	-	-	*C. cinerea*	4.05	-	-	-	-	-	-	-	-
NAC-6004	-	-	*R. vinctus*	1.62	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nithyashree	*E. sorghinum*	8.1	*S. zeae*	9.72	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bio-9637	-	-	*S. zeae*	8.91	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CM-500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*F. fujikuroi*	34.02	-	-

*1: Isolates
*2: Percent colonization

Screening of fungal endophytes against *C. partellus*

The mortality (%) and mycosis (%) of larvae were observed after 10 days of treatment (Table. 2). Among the fungal endophytes tested,

significant differences were observed in the larval mortality against maize stem borer, *C. partellus*. *Cladosporium oxysporum* and *Rigidoporus vinctus* isolates showed 6.67 and 10% mortality respectively. Rest of the isolates showed 0% mortality of *C. partellus*.

Table 2. Pathogenicity of fungal endophytes against *C. partellus*

Sl no	Isolates	GenBank accession number	Mortality (%)
1	*Acremonium zeae*	KU158874	0.0
2	*Cladosporium oxysporum*	KU158864	6.67
3	*Colletotrichum boninense*	KU158869	0.0
4	*Colletotrichum gloeosporioides*	KU158870	0.0
5	*Coprinopsis cinerea*	KU158876	0.0
6	*Curvularia lunata*	KU158873	0.0
7	*Epicoccum sorghinum*	KU158867	0.0
8	*Epicoccum sorghinum*	KU158866	0.0
9	*Fusarium fujikuroi*	KU158871	0.0
10	*Gibberella moniliformis*	KU158868	0.0
11	*Nemania sp.*	Yet to be submitted	0.0
12	*Nemania sp.*	Yet to be submitted	0.0
13	*Penicillium sp.*	KU158878	0.0
14	*Rigidoporus vinctus*	KU158872	10.0
15	*Sarocladium zeae*	KU158865	0.0
16	*Sarocladium zeae*	KU158875	0.0
17	*Scopulariopsis gracilis*	KU158877	0.0

DISCUSSION

All the plant species found a group of endophytes, endophyte protects host plants by producing substances that provide protection against phytopathogen and herbivores. In this study, 17 fungal endophytes were isolated and identified as *Acremonium zeae, Cladosporium oxysporum, Colletotrichum boninense, Colletotrichum gloeosporioides, Coprinopsis cinerea, Curvularia lunata, Epicoccum sorghinum, Fusarium fujikuroi, Gibberella moniliformis, Nemania sp., Penicillium sp., Rigidoporus vinctus,Sarocladium zeae* and *Scopulariopsis gracilis*. These fungal species also exists in other agricultural crops either endophytically or epiphytically. The fungi isolated from maize are pathogenic or nonpathogenic to maize crop; it depends on the pathogenicity factors triggered by ecological or exogenous / endogenous physiological changes (Petrini, 1991). Among the 17 fungal endophytes isolated from maize tissues, the isolate *Furarium fujikuroi* showed highest colonization with 34.02%, followed by the genera *Penicillium* with 20.01% colonization and rest of the isolates showed less colonization. This study revealed that, each maize variety has diverse group of fungal endophytes. The frequency and species of fungal endophytes are very with different part of the host plants (Suryanarayanan and Vijaykrishna, 2001). In the present study, 65% of endophytes were assembled in leaf tissues, 23% in stem tissues and 12% of endophytes were assembled in root tissues of the maize plants. In contrast, Fisher *et al*., in 1992 isolated more fungal species in stem than leaf tissues. More fungal species were isolated about 60 days old plants than 30 and 90 days old plants. This result indicates that, as the plant age increases presence of fungal species would decreased, as per McInroy and Kloeper (1995) this could be the limitation of some essential nutrients during the time of maturation of plants.

Genera *Fusarium* a ubiquitous fungi, it was routinely isolated from other host plants like, wheat and soybean. This genus consists of both

phytopathogenic and nonpathogenic group of species. The phytopathogenic group of species affects a broad range of hosts by causing foliar necrosis, root rot, vascular wilting and yellowing (Picco *et al*., 2011). Some nonpathogenic species like, *F. oxysporum* induce host resistance through a combination of antibiosis/mycoparasitism and inducing plant defense mechanism (Marciano *et al*., 2005). However, in the present study, *F. fujikuroi* (synonym *G. fujikuroi*) isolated from maize not showed pathogenicity against *C. partellus*. According to Donald *et al*., (2005), *A. zeae* functions as protective endophytes of maize and it is not identified as causing agent of ear and kernel rots of maize. No reports have been showed that, *A. zeae* isolated from maize produces any metabolites that toxic to animals and plants. *A. zeae* isolated from maize kernel showed antagonistic to kernel rotting and mycotoxin producing fungi *Aspergillus flavus* and *Fusarium verticillioides* by producing metabolites like, pyrrocidines A and B. In the present analysis, *A. zeae* isolated from maize did not have any pathogenic effect on maize stem borer, *C. partellus*. *C. gloeosporioides* and *C. boninense* are the two strains isolated from healthy maize tissues. These isolates did not show any pathogenicity against *C. partellus*. *Colletotrichum spp.* was also isolated from banana, ginger, *Euphatorium thymifolia*, soybean, longan, mango and *Draceana sanderiana* (Photita *et al.,* 2005). According to Bailey and Jeger (1992) and Lenné (1992) *Colletotrichum* species cause anthracnose, it can cause significant damage in a large group of crops such as cereals, coffee and legumes. It also causes post harvest anthracnose disease in banana and mango (Photita *et al.,* 2005).

Cladosporium, one of the largest genera of hypomycetes, these species is most common fungi isolated from the environment. Most of the species are plant pathogens, while some are commonly encountered as contaminants. In only few incidences, *C. oxysporum* exists as endophytes from pine trees (Narayan Chandra Paul and Seung Hun Yu, 2008). Bioformulation of endophytic fungus *C. oxysporum*, isolated from stems of *Euphorbia bupleuroides* subsp. *luteola* (Kralik) Maire, was tested for their aphicid activity against the black bean aphid *Aphis fabae*. Significant mortality of aphid *Aphis fabae* was observed (Bensaci *et al*., 2015). In this investigation, *C. oxysporum* isolated from maize have shown the 6.67% of mortality of stem borer, *C. partellus*. *G. moniliforme* (synonym *F. verticilliodes*; *G. fujikuroi*), a facultative fungal endophyte, which produces mycotoxin like, fumonisin B1 and B2 in maize. The symptomless association of these fungi with maize was firstly reported by Leonian in 1932 (Bacon *et al*., 2001). The community of fungal endophytes associated with maize and their pathogenicity against borer pest, *C. partellus* were studied in the present investigation. Furthermore, studies should be focus in analysis and development of an efficient strategy to control *C. partellus* based on this fungal endophytes.

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REFERENCES

1. Abbott, W. S. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*., 1925; **18**: 265.
2. Andrea Porras-Alfaro., Paul Bayman. Hidden Fungi, Emergent Properties: Endophytes and Microbiomes. Annu. Rev. *Phytopathol*., 2011; **49**:291-315.
3. Bacon,. Ida E. Yates,. Dorothy M. Hinton,. Filmore Meredith. Biological Control of *Fusarium moniliforme* in Maize. Environmental Health Perspectives,. 2001; **109**.
4. Ballal, Chandish R., Kumar., Pradyumn., Ramani. S. Laboratory evaluation, storability and economics of an artificial diet for rearing *Chilo partellus* (Swinhoe) (Lepidoptera:Pyralidae). *Journal of Entomological Research*., 1995; **19**(2):135-141.
5. Bensaci,. Harzallah Daoud,. Nadia Lombarkia,. Khamsa Rouabah. Formulation of the endophytic fungus *Cladosporium oxysporum* Berk. & M.A. Curtis, isolated from *Euphorbia bupleuroides* subsp. *luteola*, as a new biocontrol tool against the black bean aphid (*Aphis fabae* Scop.). *Journal of Plant Protection Research*., 2015; **55**(1).
6. Carroll, G. C., Carroll, F. Studies on the incidence of coniferous endophytes in the Pacific Northwest. *Canadian Journal of Botany*,. 1978);

5: 3034-3043.

7. Clement, S.L., Kaiser, W.J., Eichenseer, H.,. *Acremonium* endophytes in germplasms of major grasses and their utilization for insect resistance. In: Bacon, C.W., White, J. (Eds.), Biotechnology of Endophytic Fungi of Grasses., 1994. *CRC Press, Boca Raton, FL*, pp.185–199.
8. Dingle, J., McGee, P.A. Some endophytic fungi reduce the density of pustules of *Puccinia recondita f. sp. tritici* in wheat. *Mycological Research.*, 2003; **107**: 310-316.
9. Donald T. Wicklow,. Shoshannah Roth,. Stephen T., Deyrup., James B. Gloer. A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycological Research*., 2005; **109**(5): 610–618
10. E. Quesada-Moraga,. B.B. Landa,. J. Mun oz-Ledesma,. R.M. Jime´nez-Dia z., C. Santiago-A lvarez. Endophytic colonisation of opium poppy, *Papaver somniferum*, by an entomopathogenic *Beauveria bassiana* strain. *Mycopathologia*., 2006; **161**: 323-329.
11. Fisher. P. J., Petrini .O., Lappin Scott. H. M. The distribution of some fungal and bacterial endophytes in maize *(Zea mays* L.). *New Phytologist.*, 1992; **122**: 299-305.
12. Fu-kang Gao,. Chuan-chao Dai., Xiao-zhen Liu. Mechanisms of fungal endophytes in plant protection against pathogens. *African Journal of Microbiology Research*., 2010; **4**(13):1346-1351.
13. Leonian LH. The pathogenicity and the variability of *Fusarium moniliforme* from corn. West Virginia Agr Expt Sta Bull., 1932; **248**: 1–16.
14. Marciano R. Rubini,. Rute T., Silva-Ribeiro,. Alan W. V. Pomella,. Cristina S. Maki,. Welington L. Araújo, Deise R. dos Santos., João L. Azevedo. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom Disease. *International Journal of Biological Sciences*., 2005; **1**: 24-33.
15. McInroy. J. A., Kloepper. J. W. Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant and Soil*,. 1995; **173**: 337-342.
16. Nur Amin. Diversity of endophytic fungi from root of Maize var. Pulut (waxy corn local variety of South Sulawesi, Indonesia). *International Journal of Current Microbiology and Applied Sciences.*, 2013; **2**(8): 148-154.
17. Narayan Chandra Paul., Seung Hun Yu. Two Species of Endophytic *Cladosporium* in Pine Trees in Korea. *Mycobiology*., 2008; **36**(4) : 211-216
18. Petrini, O. Fungal endophytes of tree leaves. In: *Microbial Ecology of the Leaves* (Ed. by]. Andrews & S. S. Hirano), 1991; pp. 179-197. Springer Verlag, New York.
19. Photita, W., Taylor, P.W.J., Ford, R., Hyde, K.D., Lumyong, S. Morphological and molecular characterization of Colletotrichum species from herbaceous plants in Thailand. *Fungal Diversity.*, 2005; **18**: 117-133.
20. Picco A.M,. Angelini P,. Ciccarone C,. Franceschini A,. Ragazzi A,. Rodolfi M,, Varese GC,. Zotti M. Biodiversity of emerging pathogenic and invasive fungi in plants, animals and humans in Italy. *Plant Biosystems*., 2011; **145**: 988-996.
21. Safavi. S. A., Kharrazi.A., Rasoulian. Gh., R., Bandani. A. R. Virulence of some isolates of entomopathogenic fungus, *Beauveria bassiana* on *Ostrinia nubilalis* (Lepidoptera: Pyralidae) larvae. *Journal of Agricultural Science and Technology.*, 2010; **12**: 13-21.
22. Saikkonen K,. Faeth SH,. Helander M,. Sullivan TJ. Fungal endophytes: a continuum of interactions with host plants. Annu. Rev. *Ecol. Syst*., 1998; **29**: 319-343.
23. Suryanarayanan.T.S., Vijaykrishna. D. Fungal endophytes of aerial roots of *Ficus benghalensis. Fungal Diversity.*, 2001.