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RESEARCH ARTICLE

Effect of fungicides on association of arbuscular mycorrhiza fungus *Rhizophagus fasciculatus* and growth of Proso millet (*Panicum miliaceum* L.)

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Abstract

The detrimental effects of fungicides on non-target beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi are of interest to agriculture. *Rhizophagus fasciculatus* was found to be predominant (21%) AM fungus in studied soil compared to other species (2-9%). Hence, we have conducted a study to evaluate the potential effects of fungicides Benomyl (Methyl [1-[(butylamino) carbonyl]-1H-benzimidazol-2-yl] carbamate), Bavistin (methyl benzimidazol-2-ylcarbamate), Captan ((3a*R*,7a*S*)-2-[(trichloromethyl) sulfanyl]-3a,4,7,7a–tetra hydro-1*H*-isoindole-1,3(2*H*)-dione and Mancozeb (manganese ethylene-bis(dithiocarbamate) (polymeric) complex with zinc salt) on association of *R. fasciculatus* with Proso millet (*Panicum miliaceum* L.), an emerging drought-resistant crop that represent a cheap source of nutrients for human in developing country. The results of this study showed significant ($P \leq 0.05$) higher AM colonization (69.7%), spore density (193 spores), plant growth (both lengths and weights of shoots and roots) and grain yield (154 grains per panicle) in mycorrhizal Proso millet plants treated with Captan compared to other fungicides and untreated controls. In contrast, Benomyl had adverse effect in all parameters measured (45.3% AM colonization, 123 spores, 105 grains per panicle, etc.). Our results also showed that AM colonization significantly improve growth and grain yield of Proso millet plants compared with non-mycorrhizal plants. Therefore, the present study demonstrated that Proso millet crops could be improved with native AM fungal inoculation, however, the type of fungicide applied in soil and its effect on plant performance must be seriously considered.

Keywords: Arbuscular mycorrhizal fungi, fungicide, plant growth, Proso millet, *Rhizophagus fasciculatus*

1. Introduction

In modern crop systems, fungicides are extensively used in order to control or to eliminate fungal phytopathogens. However, fungicides applied on soils not only affect the targeted phytopathogens but also can affect autochthonous soil microorganisms including those that are beneficial to plant growth, such as arbuscular mycorrhizal (AM) fungi, which are actively involved in plant nutrient acquisition, plant protection against soil-borne pathogens and improvement of soil structure (Barea *et al*., 2002; Jeffries *et al*., 2003; Arriagada *et al*., 2012; Tanwar *et al*., 2013) [1, 2, 3, 4]. The beneficial effects of AM fungi on host plant growth and development can be hampered by the wide spread use of fungicides in agricultural systems (Trappe *et al*., 1984) [5]. Studies have showed that the length of external hyphal decreased followed of fungicide treatments with the concomitant reduction of phosphorus (P) content in plants (Kurle and Pfleger, 1994; Kling and Jakobsen, 1997) [6, 7]. Similarly, the long-term fungicide benomyl applications affected mycorrhizal root colonization in tallgrass prairie, and indirectly influencing soil biota and nutrient availability in soils (Smith *et al*., 2000). In contrast, significant impacts of the fungicides azoxystrobin, tebuconazole and chlorothalonil on soil microbial populations was not observed by Bending *et al*., (2007) reporting just a minor effect on fungal communities revealed by PCR-DGGE techniques based on 18S rRNA genes. Pasaribu *et al*., (2013) recently reported that growth and P content in peanut plants was improved by *Glomus mosseae* inoculation, but the application of alachlor fungicide significantly reduced the plant growth, in contrast, the plant growth was unaffected by glyphosate application. However, information on the effects of fungicides on AM colonization and plant growth is still limited, particularly in emerging crops with tolerance to global climate change.

Proso millet (*Panicum miliaceum* L.) is a drought-resistant crop domesticated thousands of years ago and currently cultivated in North America, Africa and Asia. It is essentially a crop of the temperate regions, but is also grown in the sub-tropics and on high ground in tropical winters. During last few years, Proso millet have raised attention because its grains are nutritionally superior to rice and wheat, and represent a cheap source of nutrients (proteins, minerals and vitamins) for human nutrition in developing country (Saleh *et al*., 2013). Based on content of phenolic compounds, studies have also indicated the potential contribution of Proso millet to disease risk reduction and health promotion in human (Shahidi and Chandrasekar, 2013). On the other hands, Proso mill*et al*so exhibits desirable characteristics as an alternative crop to fuel production because is a quick growing short duration cereal with low irrigation requirements, resistant to pests and diseases and it can be cultivated throughout the year (Rose and Santra, 2013). However, the utilization of Proso millet grains as food source and fuel production is still limited due to lack of our knowledge for ecology, cultivation and processing technologies at a commercial scale. Based on the extensive use of fungicide in crops, the importance of AM fungi in the uptake and transference of nutrients in plants as well as the potential of Proso millet as source of food and phenolic compounds for human, the main goal of the present study was to assess the effect of fungicides on the association of AM fungi and growth of Proso millet.

2. Materials and Methods

2.1. Soil and fungal spore collection

Red sandy loam soil (0-20 cm) for the potting experiments was collected from agricultural lands of Manasur in Dharwad district (15.5ºN 75.0ºE; India). The soil was air-dried, sieved (2 mm) then stored in gunny bags until used. Soil texture, organic carbon (C) and available P of soil were determined according to methods described by Bouyoucos (1962), Walkley and Black (1934) and Olsen *et al*., (1954), respectively. The number of AM fungal spores in soil was determined according to described by Gerdemann and Nicolson (1963) and identified following the current taxonomic criteria (Schenck and Perez, 1990; Schüßler and Walker, 2010; Oehl *et al*., 2011) and information taken from International Collection of Vesicular-Arbuscular Mycorrhizal Fungi (INVAM) from West Virginia University (http://invam.wvu.edu/). *Rhizophagus fasciculatus* was found to be the predominant fungus in soil and selected for inoculation in potting experiments.

2.2. Pot experiment

A pot experiment was conducted to evaluate the effects of commercial fungicides on root colonization by *R. fasciculatus* and growth of Proso millet plants. Seeds of proso millet were obtained from the Krishi Vigyan Kendra of Dharwad Agricultural University, Hanumanamatti, Haveri, Karnataka, India. The seeds were surface sterilized in 2% mercuric chloride solution for 2 minutes and washed free of sterilant using distilled water prior to sowing. The AM fungus (*R. fasciculatus*) inoculum was prepared from spores isolated from the field soil using sorghum (*Sorghum bicolor*) as the host in the Microbiology Laboratory, P.G. Department of Studies in Botany, Karnatak University, Dharwad (15°26′28.5″N, 74°59′2.1″E), India. The AM fungal inoculum (25g pot^{-1}) containing chopped mycorrhizal root bits, soil hyphal fragments and spore clusters (315-375 spores per 25 g) were layered 5 cm below the soil surface in each pot involving AM fungi. Twenty five grams of sterilized (120 °C, for 45 minutes) AM fungal inoculum was added to uninoculated treatments. Four seeds of proso millet were sown in 30 cm diameter earthen pots containing 15 kg of unsterile field soil. The seedlings were thinned to two per pot one week after germination.

Four different fungicides Benomyl (Methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl] carbamate), Bavistin (methyl benzimidazol-2-ylcarbamate), Captan ((3a*R*,7a*S*)-2-[(trichloromethyl) sulfanyl]-3a,4,7,7a–tetrahydro-1*H*-isoindole-1,3(2*H*)-dione) and Mancozeb (manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salt) were purchased from Indian agrochemical companies (BASF India Ltd., and Excel Crop Care Ltd.). The fungicidal treatments were established according to doses used in crops at field level in India with modifications described by Laird and Addicott (2008) and calculated according to the following formula: $N = TP/A_i$. Where, N represents the amount of fungicide required, T represents the total spray fluid required, P represents the percentage (%) strength required and A_i represents the given percentage strength of formulated fungicide. A completely random experimental design was adopted and the fungicide treatments were as follows: plants inoculated with *R. fasciculatus* (Mycorrhizal), plants inoculated with *R. fasciculatus* and treated with Bavistin (Mycorrhizal+Ba), plants inoculated with *R.*

fasciculatus and treated with Captan (Mycorrhizal+Ca), plants inoculated with *R. fasciculatus* and treated with Mancozeb (Mycorrhizal+Ma) and plants inoculated with *R. fasciculatus* and treated with Benomyl (Mycorrhizal+Be). Pots containing uninoculated plants and untreated with any fungicide was also included as controls (Non-mycorrhizal). The fungicides treatments were given once in a month till harvest.

2.3. Plant growth, AM fungal colonization, spore number, and grain yield

Plants were grown under greenhouse conditions with temperatures ranging from 25±3°C day-time to 15±3 °C night-time, a 16:8 h light:dark photoperiod, and a relative humidity of 80-90%. The plants were irrigated manually with tap water as needed during the experiment (judged by weighing pots). Every fortnight, 10 mL of Hoagland's nutrient (Hoagland and Arnon, 1950) solution minus P (101.10 g L^{-1} KNO_3, 246.49g L^{-1} $MgSO_4$. $7H_2O$, 1.864g L^{-1} KCl, 0.773g L^{-1} H_3BO_3, 0.169g L^{-1} $MnSO_4.H_2O$, 0.288g L^{-1} $ZnSO_4$. $7H_2O$, 0.062g L^{-1} $CuSO_4.5H_2O$, 0.040g L^{-1} H_2MoO_4, 30g L^{-1} NaFeEDTA) was given to all the experimental plants.

The proso millet plants (3 plants per treatment) were harvested at 60 days after sowing (DAS), to determine the effect of fungicides on proso millet growth by measuring the following parameters: plant height, fresh and dry weight of plant biomass (root and shoot), and root length. Before harvest plant height was measured manually using stainless steel ruler with measurement taken from the collar (at soil surface) to the shoot apex. At each harvest fresh weight of shoot and root were measured accordingly. The roots and shoot sample was oven dried at 72°C for 48 h and weighed using electrical balance. Before drying, a fraction of weighed roots was collected and AM colonization was determined using the method described by Giovanetti and Mosse (1980) after clearing and staining (Phillips and Hayman, 1973). The AM fungal spores in mycorrhizosphere soils were isolated from the substrates by wet sieving and decanting technique and enumerated as per Channabasava and Lakshman (2013). Root length was measured using a line intersect method (Newman, 1966). Root length was estimated by the formulae below as described by Tennant (1975): Root length = N×C. Where, N: The total number of intersections and C: conversion factor (0.7857).

The number grain yield (number of grain per panicle and weight of 100 grains) was determined according to method described by Ross (1971) as follow: seeds were separated from panicle, cleaned manually and weighed on electronic balance.

2.4. Statistical analysis

The data were analysed by a one-way analysis of variance (ANOVA), and comparisons were carried out for each pair with Duncan's multiple range test using SPSS statistical software (SPSS Inc., Chicago, IL, USA). All treatments were carried out in triplicate, and the values are given as means ± standard errors. Differences were considered to be significant when the *P* value was less than or equal to 0.05.

3. Results and Discussion

3.1. Soil and fungal spore collection

The soil properties analysis revealed a pH of 6.89, electrical conductivity (Ec) of 0.24, 0.65% of organic C, 0.29% of available P, 0.33 $cmol_{(+)}$ kg^{-1} of exchange cation capacity, 20.87% of clay, 16.79% of silt, 28.00% of coarse silt, and 28.94% of fine sand. The number of AM fungal spores in the native soil was ~124 spores

100 g^{-1} soil, including spores belonging to *Acaulospora laevis, A. spinosa, Archeospora trappei, Funneliformis caledonium, Gigaspora rosea, Glomus aggregatum, G. albidum, G. delhiense, G. globiferum, G. macrocarpum, Redeckera fulvum*, *Rhizophagus fasciculatus, R. clarus, Sclerocystis dussii, S. pubescens*, and *Scutellospora calospora* species. *R. fasciculatus* was predominant fungus in soils (26 spores in 100 g of soil) representing around 21% of total spores collected (Figure 1). The number of occurrence of other fungal species ranged 2 to 9% of total spores collected. Respect to higher occurrence of *Rhizophagus fasciculatus*, it has been postulated that dominance of specific genera of AM fungi in specific environment can be attributed to various environmental factors, such as soil physico-chemical properties, plant morphology, compatibility between host plants and AMF species, dispersal of fungi, among others (Muthukumar and Udaiyan, 2000; Vyas, 2005; Nasim and Bajwa, 2005; D'Souza and Rodrigues, 2013).

Table 1. Effect of fungicides Bavistin (Ba), Captan (Ca), Mancozeb (Ma), and Benomyl (Be) on percent of arbuscular mycorrhizal colonization (AMC), mycorrhizal spore number (MSN) and grain yield of mycorrhizal Proso millet plants.

Treatments	AMC (%)[a]	MSN[b]	Grain yield	
			No. of grains per panicle	**100 grains weight (g)**
Non-mycorrhizal	N.D.	N.D.	107.33±1.76 cd	0.45±0.00 c
Mycorrhizal	61.3±0.0* ab	172±1.7 b	123.67±2.40 b	0.55±0.02 b
Mycorrhizal+Ba	50.3±1.2 d	161±1.7 cd	114.67±1.76 c	0.46±0.01 c
Mycorrhizal+Ca	69.7±0.7 a	193±2.2 a	154.34±4.48 a	0.64±0.02 a
Mycorrhizal+Ma	56.7±1.2 c	167±1.2 c	125.33±1.45 b	0.55±0.02 b
Mycorrhizal+Be	45.3±0.3 e	123±1.9 e	105.02±1.73 d	0.45±0.02 c

[a]Percent of AMC in relation to total root length

[b]MSN in 25 g of mycorrizosphere soil

N.D.: not detected.

*Different letters in the same column denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Duncan's multiple range test using SPSS statistical software).

3.2. AM fungal colonization and spore number

The percent of mycorrhizal colonization by *R. fasciculatus* was significantly ($P \leq 0.05$) reduced in mycorrhizal plants treated with fungicides Bavistin (50.3%), Mancozeb (56.7%) and Benomyl (45.3%) compared with control without fungicide treatments (61.3%) (Table1). Similarly, significant ($P \leq 0.05$) lower number of spores in mycorrhizosphere soil were registered in mycorrhizal plants treated with (spores per 25 g soil): Benomyl (123) followed by Bavistin (161) and Mancozeb (167), compared with control without fungicide treatments (172) (Table 1). In contrast, in mycorrhizal plants treated with Captan showed highest values of mycorrhizal colonization (69.7%) and spore number (193 spores) in mycorrhizosphere soil.

3.3. Plant growth and grain yield

In the present experiment, the AM colonization significantly improved the plant growth compared with

non-mycorrhizal plants (Table 2). Thus, mycorrhizal plants showed a significant higher ($P \leq 0.05$) fresh weight of shoots (13.1 g) and roots (2.5 g) compared with non-mycorrhizal plants (3.6 g of shoots and 0.5 g of roots). In the same way, mycorrhizal plants showed a significant higher ($P \leq 0.05$) dry weight of shoots (4.3 g) and roots (1 g) compared with non-mycorrhizal plants (1.5 g of shoots and 0.2 g of roots). In relation to the effect of fungicides treatments on plant growth, significant ($P \leq 0.05$) lower fresh and dry weights were registered in mycorrhizal plants treated with Benomyl followed by Bavistin and Mancozeb, compared with control without fungicide treatments. It is noteworthy that significant ($P \leq 0.05$) higher fresh (13.9 g of shoots and 2.8 g of roots) and dry (2.8 g of shoots and 1 g of roots) weights were observed in mycorrhizal plants treated with Captan compared with other treatments and controls. The effects of fungicide treatments on plant weights were coincident with plant lengths. Thus, mycorrhizal plants showed a significant higher ($P \leq 0.05$) lengths of shoots (26.7 cm) and roots (17.22) compared with non-mycorrhizal plants (13.3 cm of shoots and 11.3 cm of roots) (Table 2). Significant ($P \leq 0.05$) higher lengths were observed in mycorrhizal plants treated with Captan (27.6 cm of shoots and 18.4 cm of roots) compared with other treatments (ranging 18.1-24.3 cm of shoots and 12.3-14.6 cm of roots) and controls.

The number and weight of grains was also affected in mycorrhizal plants treated with fungicides. Mycorrhizal plants treated with Captan showed higher number of grains per panicle (154 grains) compared with other fungicide treatments. Whereas reduced number of grains per panicle (105 grains) was observed when plants were treated with Benomyl. Lesser negative effect was recorded with other two fungicides treatment but the number of grains per panicle was higher when compared to non-mycorrhizal plants (107 grains) (Table 2). In relation to the weight of 100 grains, the results showed a reduced weight in plants exposed to Benomyl treatment (0.45 g) over the remaining fungicide treated plants. The highest weight of 100 grains was recorded in plants treated with fungicide Captan (0.64 g) and control plants with only AM fungus inoculation (0.55 g) (Table 2).

Table 2. Effect of fungicides Bavistin (Ba), Captan (Ca), Mancozeb (Ma), and Benomyl (Be) on growth of mycorrhizal Proso millet plants.

Treatments	Plant length (cm)		Shoot weight (g)		Root weight (g)	
	Shoot	**Root**	**Fresh**	**Dry**	**Fresh**	**Dry**
Non-mycorrhizal	13.33±1.07* bc	11.31±0.15 f	3.6±0.4 e	1.8±0.7 e	0.5±0.0 e	0.2±0.0 f
Mycorrhizal	26.71±0.39 ab	17.22±0.25 b	13.1±0.7 b	4.3±0.6 b	2.5±0.6 b	1.0±0.7 a
Mycorrhizal+Ba	24.34±1.46 abc	14.59±0.24 d	12.8±0.3 bc	4.0±0.0 bc	2.0±0.7 bc	0.7±0.0 c
Mycorrhizal+Ca	27.60±1.49 a	18.40±0.41 a	13.9±0.3 a	4.7±0.0 a	2.8±0.3 a	1.0±0.7 a
Mycorrhizal+Ma	23.76±0.26 bc	16.32±0.30 c	12.0±0.7 bc	3.9±0.5 c	2.0±0.3 c	0.7±0.1 cd
Mycorrhizal+Be	18.11±0.93 c	12.34±0.19 e	5.5±0.0 d	2.1±0.0 d	1.7±0.0 d	0.3±0.0 e

*Values represent mean ± standard error (average of three repeats). Different letters in the same column denote significant difference ($P \leq 0.05$, comparisons of means were carried out for each pair with Duncan's multiple range test using SPSS statistical software).

The effect of fungicides on AM colonization and their effectiveness on plant growth are scares and controversial (Smith and Read, 1990; Zangaro and Moreira, 2010). In this study, we demonstrate that fungicide treatments can significantly affect the root colonization by *R. fasciculatus* and growth of Proso millet plants. Scientists have stated that fungicides affect the AM symbiosis with the host plant in different manners: negatively, neutrally and positively (Samarbhakhsh *et al*., 2009). In general, experimental results showed that mycorrhizal plants treated with Benomyl produce lower AM fungi colonization and spore number in rhizosphere soil compared with other fungicides used. Suppressive effect on the activity of AM fungi by addition of Benomyl in soil had been reported by O'Connor *et al*., (2009) and Abbasian *et al*., (2012). This reduction may be attributed to the effect of Benomyl on proteins of cell membrane and cellular respiration of mycorrhizal fungal (Wasif and Laga, 2009). Benomyl fungicide also inhibits the formation and development of arbuscules and reduces the efficiency of the inoculation of plants (Kjøller and Rosendahl, 2000). Similarly, Bavistin and Mancozeb had a negative effect on the roots AM colonization and spore number when they were applied. The detrimental effects with other fungicides (i.e. iprodione, metalaxyl) on mycorrhizal colonization of diverse plants (*Citrus sinensis* L., *Allium porrum* L.) have also been reported (Gange *et al*., 1990; Carrenho *et al*., 2000). In contrast, Captan fungicide improved AM fungi colonization of roots and spore number in mycorrhizosphere soil. Burrows and Ahmed (2007), reported that the vesicle formation of AM fungi in roots was significantly higher when captan and tebuconazole+metalaxyl fungicides were applied in maize seeds. Similarly, metalaxyl fungicide treatments enhanced the AM colonization of maize plants inoculated with AM fungus (Seymour *et al*., 1994) and can stimulate the development of AM fungal mycelium in pineapple (Guillemin and Gianinazzi , 1992). In relation to varied effect of fungicides on AM colonization and spore number in rhizosphere soils, the differences could be attributed to sensitivity of *R. fasciculatus* toward diverse fungicides applied as reported for *Glomus* species (Kjøller and Rosendahl, 2000). Inchal (2002) observed similar results while studying the effect of different fungicides on AM fungi associated with a wild variety of caster under experimental conditions.

In relation to plant growth and grain yield, our results clearly showed the beneficial effect of AM colonization on plant growth compared with non-mycorrhizal plants. It widely known the AM fungi are the most important microbial symbioses for the majority of plants improving their nutrient uptake, growth, health and productivity (Grover *et al*., 2011; Berendsen *et al*., 2012). Recently, we have reported that the inoculation of AM fungus and the application of mine spoil and fly ash (2%) significantly increased the growth of mycorrhizal Proso millet and Kodo millet plants respectively (Channabasava and Lakshman, 2012; Channabasava *et al*., 2015). Respect to the varied effect of fungicide treatments on plant growth, studies have described that addition of fungicides resulted in substantial changes in plant species composition and growth of plant species in dry grassland (Dostálek *et al*., 2013). Previous studies showed that the application of carbendazim fungicide decrease the growth of perennial frobs but increase growth of graminoids (*Brachypodium pinnatum*, *B. erectus* and *Carex flacca*).

4. Conclusion

The results show that fungicide treatments affect the root colonization by *R. fasciculatus* and growth

of Proso millet plants. Treatments with Benomyl, followed by Bavistin and Mancozeb, significantly decrease the root colonization, spore number, plant growth and grains yield of mycorrhizal plants compared with mycorrhizal plants without fungicide treatments. In contrast, Captan significantly increase the root colonization, spore number, plant growth and grains yield of mycorrhizal plants compared with mycorrhizal plants without fungicide treatments. Plant growth and grain yield was also increased in mycorrhizal plants compared with non-mycorrhizal plants. The present study demonstrated that Proso millet crops could be improved by inoculation with beneficial native arbuscular mycorrhiza fungi, such as *Rhizophagus fasciculatus*. However, the type of fungicides applied in soil where the plant are grow must be seriously considered because to the varied effect on the AM fungal symbiosis and plant growth.

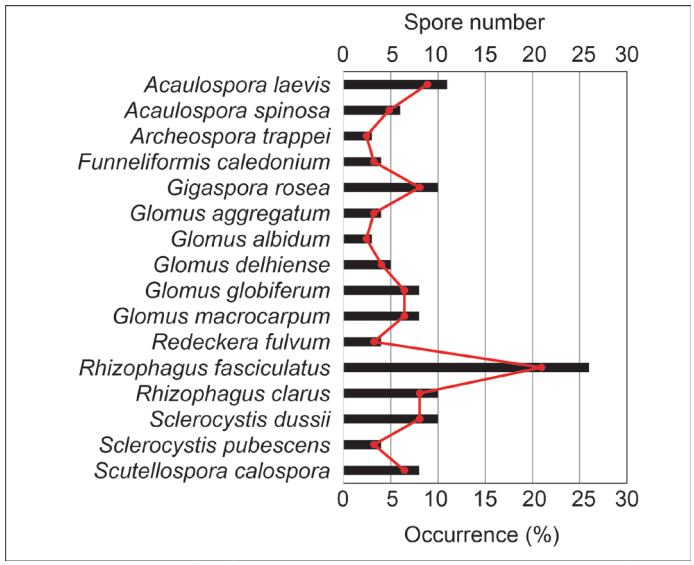

Figure 1. Density (black bars) and occurrence (red line) of arbuscular mycorrhizal fungal species in 100 g of rhizosphere soil.

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