AFPP – 5TH INTERNATIONAL CONFERENCE ON ALTERNATIVE METHODS OF CROP PROTECTION
LILLE – 11th, 12th & 13th OF MARCH 2015

ARBUSCULAR MYCORRHIZAL FUNGI ASINDUCERS OF RESISTANCE AGAINST WHEAT POWDERY MILDEW

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ABSTRACT
The use of arbuscular mycorrhizal (AM) inoculation could constitute a potential alternative method for the control of plant diseases. In the present study, wheat (*Triticum aestivum*) plants were grown in pots, under controlled conditions. The effect of phosphorus (P) concentration and the effectiveness of three different AM inocula were tested on the Orvantis wheat cultivar. After 42 days of culture, mycorrhizal (M) and non-mycorrhizal (NM) wheat plants were infected by the biotrophic fungus *Blumeria graminis* f.sp. *tritici* (*Bgt*), the causal agent of powdery mildew. Our work shows that (i) a decrease in P concentrations enhance M rates, (ii) the intermediate P/5 concentration allowed both satisfactory wheat growth and mycorrhizal rate in our conditions(iii) a decrease in P concentration results in a decrease of powdery mildew symptoms. Taken together, our results indicate that the inoculation by AM fungi induces a systemic resistance in wheat against *Bgt*. This protection level reached up to 77% with *Fm* inocula at the P/5 concentration.

Keywords: arbuscular mycorrhizal inoculation, wheat, powdery mildew, phosphorus, biocontrol.

RESUMÉ
L'utilisation de l'inoculation par des champignons mycorhiziens arbusculaires (CMA) pourrait constituer une méthode alternative potentielle de lutte contre les maladies des plantes. Dans la présente étude, des plantes de blé (*Triticum aestivum*) ont été cultivées en pots, en conditions contrôlées. L'effet de la concentration de phosphore (P) ainsi que l'efficacité de trois types d'inocula mycorhiziens, ont été testés sur le cultivar de blé Orvantis. Après 42 jours de culture, les plants de blé mycorhizés (M) et non-mycorhizés (NM) ont été infectés par le champignon biotrophe *Blumeria graminis* f.sp. *tritici* (*Bgt*), agent causal de l'oïdium. Nos résultats montrent que (i) une diminution des concentrations de P augmente les taux de M (ii) la concentration intermédiaire P/5 permets à la fois une croissance du blé et un taux mycorhizien satisfaisants dans nos conditions(iii) la diminution de la concentration en P réduit les symptômes de l'oïdium. L'ensemble de nos résultats indiquent que l'inoculation par les CMA induit une résistance systémique chez le blé contre *Bgt*. Cet effet protecteur atteint 77% avec l'inoculum *Fm* et la concentration de phosphore P/5.

Mots-clés: inoculation mycorhizienne arbusculaire, blé, oïdium, phosphore, biocontrôle.

INTRODUCTION

Wheat is the most widely cultivated cereal in the world, with more than 215 million hectares planted annually (http://www.wheatinitiative.org). About 671 million of tons are produced in 2012 (FAOSTAT,http://faostat.fao.org). Unfortunately, this crop is attacked, amongst other foliar diseases, by powdery mildew caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*), a biotrophic fungus widely distributed throughout the world, particularly in humid regions. Severe yield losses (about 30 %) can be associated with early infections of highly susceptible cultivars (http://www1.agric.gov.ab.ca). Control of this pathogen requires the intensive and systematic use of fungicides, which cause environmental and human health problems (reprotoxicity, neurotoxicity, carcinogenicity). A potential alternative control strategy is the stimulation of natural plant defenses by using plant beneficial microorganisms such as arbuscular mycorrhizal (AM) fungi. AM symbiosis occurs in more than 80% of terrestrial plants (Smith and Read,2008) and improves crop production and resistance to biotic and abiotic stresses (Pozo *et al,* 2009). The establishment of AM symbiosis involves remarkable changes in the physiology of the host plant and its potential to improve the nutrition of crops has been largely documented (Smith *et al*, 2009). Improvement of phosphorus (P) nutrition has received most attention (Gilbert, 2009). In addition, the beneficial effects of AM symbiosis in plant control disease have been mainly observed in the case of soil-borne pathogens and alleviation of damage has been shown for diseases caused by fungi (Pozo *et al*, 2010) and parasitic nematodes (Vos *et al,* 2012). Such a protective role may rely on different mechanisms and are unlikely to be exclusively dependent on an improved mineral nutrition (Liu *et al*, 2007). On the other hand, information on the effects of the AM symbiosis on pathogens attacking above-ground parts of plants is scarce and more controversial. Different mechanisms of defense were noticed. Concerning AM fungi-colonized root defense pathways, the accumulation of phenolic compounds in the plant cell wall may result in an increased lignification (Cordier *et al*, 1998). Lignification, as an essential mechanism for disease resistance (Morandi *et al*, 1984), could be explained by high activity levels of enzymes involved in its formation such as peroxidase (POX) and phenylalanine ammonia-lyase (PAL). Lignification may contribute to the reduction in pathogen proliferation directly in mycorrhizal roots (Naqvi, 2004) and defense-associated activities may participate indirectly in plant shoot resistance thanks to AM signals transferred from plant roots to aerial parts.

The objectives of the present work were to optimize the wheat mycorrhizal conditions, by studing the effect of P concentration and AM fungi inocula type on the wheat mycorrhizal rate and to investigate the impact of these conditions on the infection by *Bgt.*

MATERIALS AND METHODS

BIOLOGICAL MATERIAL,EXPERIMENTAL DESIGN AND INOCULATION BY AM FUNGI

Wheat (*Triticum aestivum*) cv. Orvantis susceptible to powdery mildew caused by *Bgt* was used in this study. Seeds were pre-germinated on moist vermiculite and then transplanted into pots containing an autoclaved substrate that consisted of sand, perlite and vermiculite (2:1:1). To obtain mycorrhizal (M) plants, the substrate was supplemented with 10 % of one of the three types of inocula:

1. *Rhizophagus irregularis* (*Ri*), (Stockinger *et al*, 2009), produced in our laboratory.
2. *Funneliformis mosseae* (*Fm*) (MycAgro Ltd, France)
3. SOLRIZE® (SZE): consisted of *Glomus sp.*(Agrauxine Ltd, France).

Non-mycorrhizal (NM) plants were supplemented with equivalent amounts of autoclaved inocula, which were free from infective AM fungi propagules in order to provide plants with the same organic

substances as AM fungi-inoculated plants. Plants were irrigated with a Hoagland's solution (Hoagland and Arnon, 1950) (50 mL/pot/week). Three (P) concentrations (P, P/5 and P/10) were tested, with P representing the average concentration of P applied in wheat field in France (P = 62 $mg.L^{-1}$ corresponding to approximately 73 $kg.ha^{-1}$ of P, Le Souder *et al*, 1998). Plantlets were grown in a controlled room culture at 12-18°C night/day, with a 12h-12h photoperiod and 70% humidity.

ESTIMATION OF MYCORRHIZAL RATE

Roots were cleared in KOH 10% for 1 h at 70 °C and stained with Trypan Blue 0.05% for 1 h at 70 °C (Phillips and Hayman, 1970). Total (T), arbuscular (A) and vesicles (V) root colonization rates were estimated according to the method of McGonigle *et al,* (1990). For each treatment, five slides containing each 20 stained root fragments were made and 3 sections per root fragment were observed under an optical microscope (x100).

PLANT GROWTH PARAMETERS

Overall, we used 3 distinct pots as biological replicates for each treatment, considering every pot as a sample (5 plants/pot). After 6 weeks of culture, different parameters of plant growth (height, leaf and stem numbers, shoot and root dry biomasses) were evaluated. The means of plant lengths and leaf and stem numbers were measured/pot, then shoot and root parts were dried in the oven at 60°C (g/pot).

PATHOGEN INFECTION

After 6 weeks of culture, NM plants and M plants were sprayed thoroughly by *Bgt* (5.10^5 spores/mL and 0.5 ml/plant), 8 days later, the number of colonies of *Bgt*/leaf was recorded.

STATISTICAL ANALYSIS

Overall, we used 3 distinct pots as biological replicates for each treatment, (5 plants/pot). Data were analyzed statistically by mean comparison by the least significant difference (LSD) test, using Statgraphics release 5.1 (Manugistic, 278 Inc., Rockville, MD, USA). Percentages were converted to arcsin values before the analysis of ANOVA and LSD test.

RESULTS AND DISCUSSION

1. EFFECT OF PHOSPHORUS CONCENTRATION ON WHEAT MYCORRHIZAL RATE

The highest mycorrhizal rates (T, A and V) were obtained, after 6 weeks of culture, with *Fm* compared to SZE and *Ri* and were estimated at about 47, 27 and 13% respectively at P/10 and 38, 17 and 16% at P/5 (Table I).This finding agrees with those previously reported by I.T.A.B group (2002), 35-40% M rate for wheat plants was reported between 6 – 7 weeks of cultivation and was described as a M rate corresponding to a well established symbiosis. Arbuscules are considered as the major site of exchange between the fungus and host (Smith, 1995) and vesicles allow the accumulation of storage products (Biermann & Linderman 1983). (Figure 1).

Our results presented in Table I showed that the low P concentrations P/5 and P/10 led to more important mycorrhizal rates with *Ri,* SZE and *Fm*, compared to the P concentration. These results are in accordance with those obtained by Ratnayake *et al*, (1978), who found that low P concentrations (0, 6 and 28 ppm) applied for 8-10 weeks increased AM colonization of *Sorghum vulgare*. Mycorrhizal rate enhancement was explained by an increase in the membrane permeability leading to the release of metabolites in sufficient amounts to sustain the initial growth and development of mycorrhizal spore germination (Ratnayake *et al*, 1978, Mohammad and Malkawi, 2004) in low P level

conditions.In addition, P acts systemically to repress symbiotic gene expression in particular genes encoding enzymes of carotenoid and strigolactone biosynthesis, and symbiosis – associated phosphate transporters, subsequently inhibiting AM colonization in the root (Breuillin *et al,*2010).

Table I. Impact of phosphorus concentrations (P, P/5 and P/10) on the M colonization of plant at 6 weeks of culture, inoculated or not by one of three AM fungi inocula types: *Ri*, SZEand *Fm*. Different letters (a, b, c) indicate statistical significant difference between mycorrhizal rates.T : total, A: arbuscules andV: vesicles.

Tableau I : Effet de la concentration en phosphore (P, P/5 et P/10) sur la colonisation mycorhizienne des plants à 6 semaines de culture, inoculés ou non par l'un des trois types d'inocula mycorhiziens: *Ri*, SZEet *Fm*. Les différentes lettres (a, b, c) indiquent une différence significative entre les taux mycorhiziens. T : total, A: arbuscules et V : vésicules.

	P concentrations			
	Inocula type		P	P/5	P/10
Mycorrhizal rate (%)	NM		0	0	0
	Ri	T	9,164 ab	18,541 c	25,482 d
		A	0 a	5,071 a	2,104 a
		V	6,652 b	15,150 c	24,467 d
	SZE	T	5,071 a	7,806 ab	13,214 bc
		A	3,587 a	1,484 a	2,967 a
		V	2,967 ab	0 a	0 a
	Fm	T	16,572 c	38,393 e	46,891 f
		A	0 a	17,014 b	26,605 c
		V	0 a	15,728 c	12,5445 c

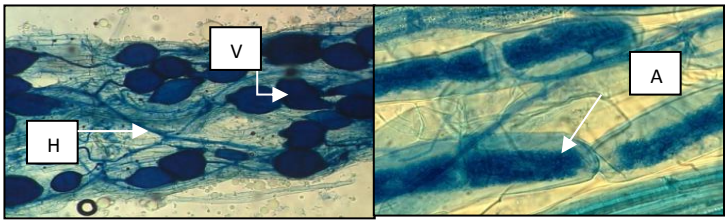

Figure I. The different structures of AM fungi: H: hyphea, A: arbuscules, and V: vesicles.
Figure I. Les différentes structures de CMA: H: hyphes, A: arbuscules, et V : vésicules.

2. EFFECT OF PHOSPHORUS CONCENTRATION AND AM FUNGI INOCULA TYPE ON WHEAT GROWTH.

The comparison between NM plant lengths for the three P concentrations showed that plants were significantly longer by 9.5% at P/5 compared to P. When we decreased P concentration, M plant lengths with *Ri* were significantly reduced at low P concentrations P/5 and P/10 by 18 and 22% respectively compared to P. In addition, plant length of M plants with SZE was reduced compared to NM plants whatever P conditions.

Concerning leaf numbers, there was no significant differences between NM plants at different P concentrations. At the lowest P/10 concentration, leaf numbers of M plants with SZE and *Fm* decreased significantly by 15 and 33% for SZE and by 17.5 and 34% for *Fm* compared to P/5 and P respectively. There was no impact of M inoculum on leaf numbers compared to NM plants for each P concentration. In contrast, reducing P concentration affected negatively plant stem numbers.

On the other hand, root biomass increased in M plants with *Ri* and *Fm* at the lowest P/10 concentration compared to NM plants. Conversely, shoot biomass was reduced for both M and NM plants compared to that observed at P/5 and P concentrations (TableII).

Table II: Effect of P concentration and M inocula on wheat growth at 6 weeks (P= 62 mgL^{-1}). Data are presented as means obtained from 5 replicates. Different letters (A, B, C) indicate statistical significant difference between NM or M plants at diffrent P concentrations, (a, b, c) indicate statistical significant difference between different traitements for each P concentration. The method used to discriminate between the means was the LSD test (P≤ 0.05).

Tableau II: Effet de la concentration de P et de l'inoculum mycorhizien sur la croissance du blé à 6 semaines (P = 62 mgL^{-1}). Les données sont présentées en tant que moyennes obtenues à partir de cinq répétitions. Les différentes lettres (A, B, C) indiquent une différence significative entre les plants NM ou M à différentes concentrations de P. Les différentes lettres (a, b, c) indiquent une différence significative entre les différents traitements au sein de chaque concentration de P. La méthode utilisée pour comparerles moyennes est le test LSD (P ≤ 0,05).

	P				P/5				P/10			
	NM	Ri	SZE	Fm	NM	Ri	SZE	Fm	NM	Ri	SZE	Fm
Plant length (cm)	42 A a	45. 4 A b	37 A c	42.2 A a	46 B b	37 B a	35 A a	41 A b	44 AB c	35 B b	31 B a	37 B b
Leaf numbers /pot	8 A ab	7AB a	9 A bc	10 A c	8 A a	8 A a	7A a	8B a	6.6A a	6B a	6B a	6.6C a
Stem numbers /pot	2 Ab	2 Ab	3 A a	3 A a	2A b	2 A b	2B b	2 B b	1B c	1B c	1C c	1C c
Root dry weight (g/pot)	0.32 A a	0.43A a	0.41A a	0.42A a	0.30 A a	0.37A a	0.32A a	0.43 A a	0.27A a	0.40A b	0.24 A a	0.42A b
Shoot dry weight (g/pot)	1.07AB a	1.17A a	1.20A a	1.63 A b	1.18A a	1.87B b	0.98AB a	1.30 AB a	0.77B a	0.74C a	0.71C a	0.83C a

The plant growth response to AM fungi colonization is described as depending on the balance between a depressor effect due to the fungal requirements, mainly photosynthesis product that provide carbon (C) for the production and maintenance of the fungal biomass (symbiosis cost), and the benefits of interaction such as a better nutritional status of the plant host (Graham, 2000). However, when AM symbiosis cost in photosynthesis products exceeds the benefits of the interaction, the plant – AM fungi relationship can turn into transient parasitism (Johnson *et al,* 1997; Pozo*et al,* 2002).

3. *EFFECT OF PHOSPHORUS CONCENTRATION AND AM FUNGI INOCULA TYPE ON WHEAT INFECTION BY Bgt.*

Our results demonstrated in NM plants a positive impact of low P concentrations where the number of colonies/leaf decreased. Indeed, *Bgt* infection rates was reduced by 30 and 33% with P/5 and P/10, respectively compared to P. Zafar in (2003), found the same impact when he used different P concenrations (P, P/2, P/4 and P/16), applied on *Gossypium hursutum* in resistance to cotton *leaf curl virus*.

All types of AM inocula (*Ri*, SZE and *Fm*) conferred a protection against wheat powdery mildew, except *Ri* M inoculation which reduced *Bgt* symptoms only at the low P concentrations P/5 and P/10. The protection rates reached 86, 70 and 74% with SZE and 84, 77and 67% with *Fm* inocula compared to (NM) control for P/10, P/5 and P, respectively (Figure 2). These results suggest a systemic defense of wheat against *Bgt* caused by AM fungi. Likewise,Pozo *et al,* (2002) tested several AM fungi and reported a bio-protective effect of *Fm* against *Phytophthora parasitica* in tomato, whereas *Ri* inoculation did not show any protection.

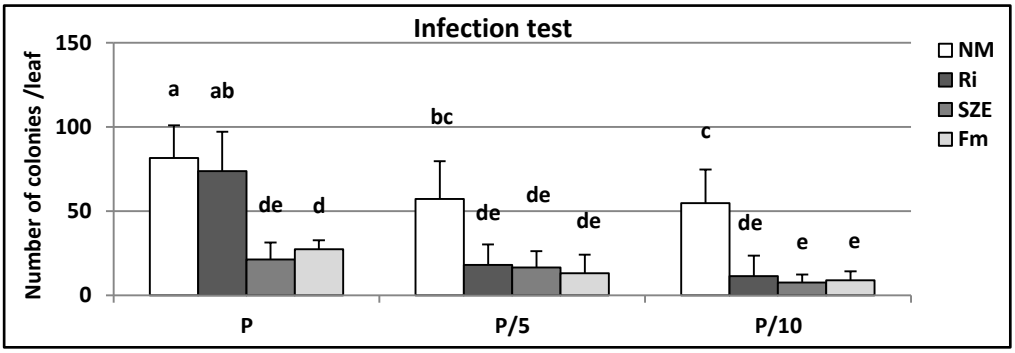

Figure 2. Impact of phosphorous concentration and Minoculation on *Bgt* infection level. Different letters (a, b, c) indicate statistical significant difference between infection rates.The method used to discriminate between the means was the LSD test (P≤ 0.05).

Figure 2. Impact de l'inoculum mycorhizien sur l'infection par *Bgt*. Les lettres différentes (a, b, c) indiquent une différence significative entre les taux d'infection. La méthode utilisée pour comparer les moyennes est le test LSD (P ≤ 0,05).

Our results showed that a high protection to *Bgt* was not associated with a high AM root colonization. Indeed, we observed that colonized wheat roots with *Fm*, *Ri* and SZE , the T mycorrhizal rates using intermediate P/5 concentration were (38, 19 and 8%) respectively with a significative difference between them (Table I). Conversely, protection rates by *Fm*, *Ri* and SZE inoculum were approximately similar 77, 67 and 70% respectively. This result is in accordance with these of Castellanos-Morales *et al,* in 2012 indicating that some AM fungi need high root colonization rates to protect plants against fungal pathogens, whereas others act already at low root colonization rates.

Most of works mentioned that AM fungus species is a key factor determining the effect of the symbiosis on the interactions with other phytopathogens because of the extent of root colonization by the AM Fungi. Reports about mycorrhizal protection against pathogens show the requirement of a well established symbiosis prior to the challenge with the parasite (Rosendahl 1985; Cordier *et al,* 1998; Slezack *et al,* 2000; Khaosaad *et al,* 2007). In contrast many authors, found that the protection against pathogens did not require a well colonisation by AM symbiosis (Caron *et al,* 1986; García-Garrido and Ocampo 1988; St-Arnaud *et al,* 1997; Kapoor 2008).

4. *EFFECT OF INFECTION BY BGT ON AM COLONIZATION RATE.*

In this experiment, we used just the intermediate P concentration (P/5) and when we compared mycorrhizal rates between non-infected mycorrhizal plants (M) and infected mycorrhizal plants (M+INF) by *Bgt*, we observed that *Fm* (T, A and V) M rates were increased with *Bgt* infection by 1.8, 3 and 2.6 folds respectively (Table III). It was proposed that the pathogen increases C or soluble sugar metabolism in infected plants. The resulting metabolites could be transferred from the host plant to the AM fungus (Li *et al,* 2013), leading to an increased AM colonization and in particular arbuscular differenciation rate. Since arbuscules are the exchange sites of the symbiosis, they regulate the delivery of P and N from the AM fungi to the host plant (Bucking and Shachar-Hill *et al,* 2005; Hammer *et al,*2011).

Table III. Effect of infection by *Bgt* on AM colonization rates at 7 weeks of culture (P/5= 12.5 mg/L). Different letters (a, b, c) indicate significant differences between AM colonization rates. *: indicate a significant difference between (M) plants and (M+INF) by *Bgt*. The method used to discriminate between the means was the LSD test (P≤ 0.05).

Tableau III. Effet de l'infection par *Bgt* sur les taux mycorhiziens à 7 semaines de culture (P/5 = 12,5 mg/L). Des lettres différentes (a, b, c) indiquent des différences significatives entre les taux mycorhiziens. *: Indique une différence significative du taux de mycorrhization entre les plants (M) et (M + INF) par *Bgt*. La méthode utilisée pour comparer les moyennes est le test LSD (P ≤ 0,05).

Inocula type	Mycorrhizal rate		
	T	A	V
Ri	6.67 a	5.33 a	0.33 a
Ri + INF	12 a	11 a	1 a
SZE	23.66 b	18 b	0 a
SZE+INF	24.66 b	17 a	1 a
Fm	36 c	12 ab	10.67 b
Fm+INF	67.33 c*	35.33 b*	26.67 b*

CONCLUSIONS

Taken together, our findings highlighted that a decreased of the P concentration led to an increased in M rate. Powdery mildew symptoms were increased when we used the hight P concentration. Meanwhile, the lowest P/10 concentration (6.2 mg/L) decreased stem numbers, and the intermediate P/5 concentration (12.5 mg/L) allowed both satisfactory wheat growth and mycorrhizal rate. In our conditions, all the AM inoculum tested (*Ri*, SZE and *Fm*) demonstrated their efficacy to protect wheat, particularly at low P concentrations. This protection suggested that AM fungi are able to induce a systemic resistance against the foliar and obligate biotrophic pathogen *Bgt.* Moreover, M development increased in the roots of M plants with *Fm* infected by *Bgt* compared to M plant without infection, suggesting that the foliar pathogen activate the metabolism of carbohydrates toward AM fungus.

For a better understanding of the mechanisms involved in AM wheat protection against *Bgt*, it will be interesting to investigate(i) enzymatic defense activities like POX andPAL, key enzymes involved in phenylpropanoid biosynthesis and/or lignine pathway(ii) cytological biomarkers including fluorescent papillae presence and fungal haustorium formation at the penetration sites of *Bgt* and (iii) defense gene expression (*i.e.* genes encoding PR-proteins such as, PR1, PAL chitinases…).

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