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Enhancing the Resistance of *Pinus roxburghii* Sarg. Seedlings against *Pythium debaryanum* R. Hesse using Vesicular-Arbuscular Mycorrhiza (VAM) and Acibenzolar-S-Methyl (ASM)

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ABSTRACT

This study was conducted to assess the likely inhibitory effects of vesicular-arbuscular mycorrhizal fungus (VAM), acibenzolar-s-methyl (Bion®), and Rhizolex® 50WP fungicide against *Pythium debaryanum* pathogen. The results showed that the inoculation with VAM was able to markedly decrease the growth of the pathogen. Bion showed high antifungal efficacy against *Pythium debaryanum* Oomycete. The seedlings of *Pinus roxburghii* that were inoculated with VAM displayed the highest growth parameters in terms of shoot height, root dry weight, and total dry weight in both seasons, followed by the treated seedlings with Bion in both seasons. The enzymatic tests revealed that mycorrhiza and Bion were able to significantly enhance levels of enzyme activities related to disease resistance, including peroxidase and polyphenol oxidase, which were clearly increased compared with the control. Mycorrhiza showed a higher effect on increasing enzyme activities related to disease resistance than Bion. Total phenols increased in all treatments as compared to the nonmycorrhizal, non-pathogen-treated control, but were highest when plants were inoculated with both the mycorrhizal fungi and the pathogen. This study recommends VAM as an inoculum for increasing the productivity performance of *P. roxburghii* seedlings and controlling the growth of *P. debaryanum*.

INTRODUCTION

Plant disease is one of the major limitations encountered the productivity of crops and reduced the availability, absorption, distribution, and use of nutrients by the plant (Romero et al., 2011). Oomycete phytopathogens caused many plant diseases, especially in subtropical and tropical regions (Bibi et al., 2018; Santra and Banerjee, 2020). Chemical fungicides are extensively used in agriculture; however, these products may cause problems, such as environmental pollution, which consequently affects human health. Several microorganisms are used as biological control agents as they have a high potential to control plant pathogens (Babbal et al., 2017). Numerous reports are dealt with the potential use of biocontrol agents as alternations for agrochemicals (Abbas et al., 2019; Saha et al., 2016; Veliz et al., 2017). However, their usage is not extensive, therefore more research are required for more effective chemical treatments.

The largest Pythiaceae genus, Pythium, contains 92 species in soil. Many of these species are saprophytes, meaning they can only grow in wet settings. A few of these species even take part in mycorrhizal relationships (Lilja, 1994; Sewell, 1981; Shearer and Smith, 2000). Many plant species seedlings are parasitized by *Pythium debaryanum*, which also spreads the deadly disease damping-off (Gomathi et al., 2011; Parveen and Sharma, 2015), and is a major issue in the field since it kills newly emerged seedlings (Dubey et al., 2020; Lamichhane et al., 2017; Majeed et al., 2018). The management of pest populations below harmful levels through the use of their own natural enemies is known as biological control (Collier and Van Steenwyk, 2004; Eilenberg et al., 2001; van Lenteren et al., 2018). Increased usage of synthetic chemicals is blamed for an increase in agricultural output (Gill and Garg, 2014). Many of these compounds, such as nematicides, herbicides, and fungicides, are not biodegradable and hence become hazardous to living things (Kavita and Geeta, 2014). Crop productivity and quality will be improved by controlling this pathogen with biocontrol agents (Hassan et al., 2021; Mohamed et al., 2021(Abd-Elkader et al., 2022; Behiry et al., 2022; Hassan et al., 2021; Mohamed et al., 2021).

Biochemical changes involved in initiating Systemic Acquired Resistance (SAR) by resistance inducers can act as markers of induced resistance (Hashem and Abo-Elyousr, 2011; Huang et al., 2016). These include the increase of phytoalexins, cross-linkages of the cell wall with lignin (Bradley et al., 1992; Sattler and Funnell-Harris, 2013;

Thangavelu et al., 2003), and an increase in certain defense-related enzymes (He et al., 2002). Peroxidase enzyme has been involved in programmed cell death, the formation of papillae, and the polymerization of lignin from monomeric lignols (He et al., 2002).

Bion® is the commercial name of Acibenzolar-S-methyl (ASM) and acts as Elicitor. ASM is the most potential SAR activator (Amini, 2015; Baysal and Zeller, 2004) and elicits the same SAR pathway which includes the same pathogenesis-related (PR) proteins as observed in SAR (Friedrich et al., 1996). Bion®, a plant health promoter in annual crops, was commercially released in some countries (Baysal et al., 2003), which protects against a broad spectrum of pathogens in several crops under field conditions (Buonaurio et al., 2002; Małolepsza, 2006; Pradhanang et al., 2005). Application of ASM suppressed Phytophthora blight on pepper caused by *Phytophther capsici* (Matheron and Porchas, 2002) and reduced other diseases caused by Oomycete phytopathogens (LaMondia, 2008; Leskovar and Kolenda, 2002). The two main cucumber diseases, powdery and downy mildews, were successfully combated systemically by ASM (Ishii et al., 2019).

The aims of this study were to investigate the effect of *Glomus mosseae* and ASM on *Pinus* damping-off disease and to study its effect on the induction of defense-related enzymes and total phenolic contents against the disease under greenhouse conditions.

MATERIALS AND METHODS

Experimental place and time

The experimental work study was conducted at the nursery of Forestry and Wood Technology Department, experimental station of the Faculty of Agriculture, University of Alexandria, Abis region, Alexandria, Egypt. The experiment was carried out during two successive growing seasons, 2019 and 2020.

***Pinus roxburghii* Sarg. host plant**

The seeds of *P. roxburghii* were collected from 28 years old tree grown at the Faculty of Agriculture, Alexandria University, Alexandria, Egypt, and planted at the nursery of the Department of Forestry and Wood Technology, Abies Station, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

Soil and seed sowing

The soil used in this study was a mixture of sand, peat, and perlite at a ratio of 1:1:1, by volume. Four surface-sterilized seeds with 10% NaOCl were sown per pot. The chemical and physical analysis of the soil can be found in previous work (Frahat and Shehata, 2021).

Table 1: Chemical and physical properties of soil

Characteristics	Value
pH (1 soil: 2.5 d.w.)	8.6
E.C. (mmohs/cm)	11.5
Anion (mq/100 g soil)	
Cl^-	103
HCO3-	2.4
SO4—	26.4
CO3--	-
Cations (mq/100 g soil)	
Mg ++	22.3
Na +	91.2
Ca ++	18.3
K +	1.9

Symbiotic agents

Vesicular arbuscular mycorrhizal fungus (VAM)

Inocula soil (medium and root debris) of *Glomus mosseae* fungus, which was obtained originally from the Experimental Station of Philipps University, Botany Department, Marburg/Lahn, Germany was used.

Pathogenic agent (*Pythium debaryanum*)

Pythium debayanum fungus strain (obtained from Plant Pathology Departments, Faculty of Agriculture, Alexandria University), which causes damping off disease.

Elicitor and Rhizolex 50WP doses

The dosage of Bion WG 50, also known as acibenzolar-S-methyl (ASM), was 500 mg/Kg soil (Amini, 2015). Rizolex-T (Tolclophos-methyl), a fungicide, was applied at a dosage of 1g/kg soil. Experimental procedure.

Seeds of *P. roxburghii* were sown in plastic pots (17.5 cm diameter), filled with ca 2.0 kg of 1:1:1 sand, peat and perlite (v:v:v), except for the upper 3 cm from the rim. After seed germination; the seedlings were irrigated every day during the first three months, then every 2 days to replenish the water consumption deficits. The average of water amount used in each irrigation was about 200 ml of tap water. Seedlings were inoculated with about 3g of a medium containing VAM each g contained ca 50 chlamydospores. Control seedlings were also inoculated, but with sterilized (previously autoclaved) inocula. Survival (%) of seedlings was recorded after inoculation. Seedling height was measured, after inoculation, then every 2 weeks intervals until the end of the experiment span in December 2019. At the same time, the seedlings were treated with Bion and Rhizolex. In addition, feeder root samples were taken 2 weeks after inoculation, then monthly to check *in situ* the symbiotic agents, if any. The procedure described above has been conducted typically in the second season, 2020.

Ultrastructural examination of infected feeder roots with VAM

Feeder-roots samples were collected, washed free from debris, cut into small pieces (3 mm length) then soaked in the chain of ethanol solution, ranging from 10 to 100% (absolute), then in xylol. The specimens were soaked in each concentration for 1 h, dried, and fixed for scanning electron microscope (SEM) examination (Frahat et al., 2018; Hayat, 1974).

Peroxidase Enzyme assay

For the determination of peroxidase, the method described by (Fehrmann and Dimond, 1967; Urbanek et al., 1991). Peroxidase was determined in non-inoculated plants after three weeks. Enzyme activity was measured by blending 5g of noninoculated and inoculated tissues in a mortar with 30 ml of 0.1 M phosphate buffer (pH 6). Extracts were centrifuged for 15 min at 4000 rpm. Dilution was made by adding 4 ml distilled water to 2 ml supernatant. Aliquots of diluted supernatant were assayed for peroxidase activity using spectronic 70 at 470 nm. The reaction mixture consisted of 1.5 ml of 0.04 M catechol solution, 1.5 ml H_2O_2 (20 volume); 1.5 ml of 0.1 M phosphate buffer (pH 6), and 0.2 ml of extract. The check treatment was similarly achieved with the exception that the extract was previously boiled. The difference in optical density between the reaction mixture and that of the check was taken as a measure of the activity of the reaction. Enzyme activity was expressed as the increase in optical density from 60 to 120 sec after the substrate was added.

Polyphenol oxidase (PPO) activity

The following assay was used to measure the polyphenol oxidase activity (Sinsabaugh, 2010): A milliliter of extract and 1 ml of buffer-prepared 10-ml 1-dihydroxyphenylalanine (DOPA) were combined. Following mixing, the absorbance at 460 nm was measured initially. The samples were submerged in a water bath at 37 °C for 1h, and the absorbance was measured at 460 nm once again. An enzyme-free substrate control was conducted concurrently. In the process of forming melanin, dopachrome, an intermediate product, is produced when DOPA is oxidized (Mayaudon et al., 1973). On leaf fragments immersed in 2 ml of buffer and 2 ml of substrate, bound polyphenol-oxidase activity was measured. Short centrifugation stages were added, and the reaction time was shortened to 15 min. The expression for polyphenol oxidase activity was absorption (A) units per gram of leaf litter. In the 2 ml reaction mix, one A unit equals an A460 of 1.000 per hour under the previously mentioned circumstances.

Determination of Total Phenols

To determine the total phenols in the collected samples we used the method of Parada et al. (2019). The reagents were added in the following order to a microtube: 15 µL of standard or extract, 750 µL of deionized water, 75 µL of Folin-Ciocalteu reagent, 300 µL of sodium carbonate 20% m/v, and 360 µL of deionized water. The solutions were incubated at 20 °C for 30 min in the dark; 250 µL of the solution was then added to a 96-well plate, and the absorbance was read at 750 nm (Parada et al., 2019). The tests were performed using chlorogenic acid as a standard, in which the concentrations of the calibration curve corresponded to 100, 200, 300, 400, and 500 mg/L.

The experimental design

Randomized complete block design (RCBD) was used in this experiment. The split-plot technique was used in analyzing the data obtained, where the main plot was for inoculation with the *Pythium debaryanum*, and the sub plot was for main treatments according to (Snedecor, 1956) the data were statistically analyzed using SAS Ver. 9.1.3 (SAS, 2007). The outline of the source of variation and the degrees of freedom is given in Table 2.

Table 2: Outline of the source of variation and its degrees of freedom the experiment used

Source of variance	d.f.
Replicates	4
A	1
Error a	4
B	3
AB	3
Error b	8
Experimental error	18
Total	31

A= inoculation with the *Pythium*, B= main treatments in terms of. Control, VAM, Bion and Rhizolex.

RESULTS

1. Mycorrhization

The scanning electron microscope examination has revealed the colonization of extrametrical hyphae of VAM of rootlet cortex cells as shown in (Fig.1 A). The surface of the feeder root of *Pinus roxburghii* infected with VAM and *Pythium debaryanum* (Fig.1 B).

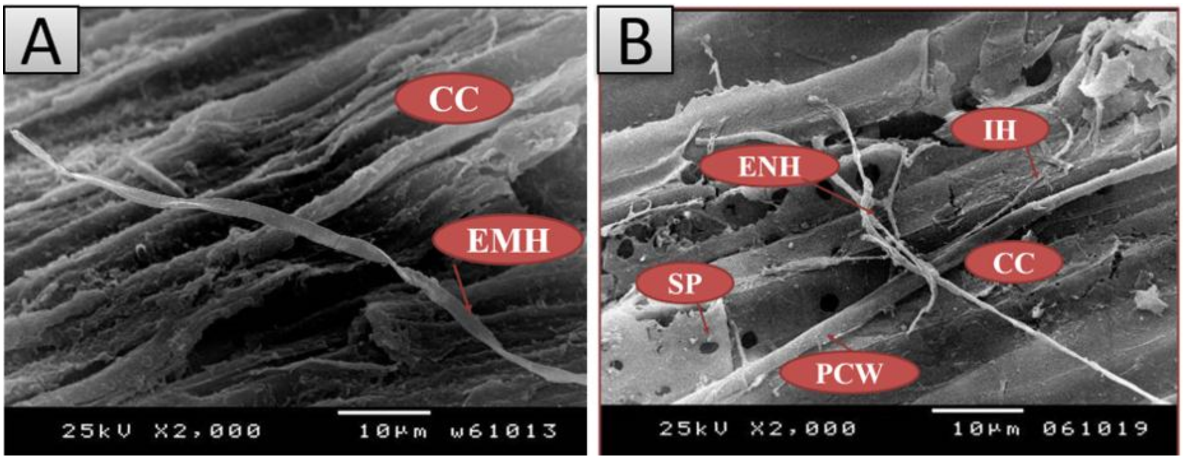

Figure 1: (A) Scanning electron micrograph (SEM) indicates extramatrical hyphae of VAM (EXM) in the cortex of feeder root of *Pinus roxburghii*. (B) Surface of the feeder root of *Pinus roxburghii* infected by VAM/ endometrical hyphae (ENH), PCW: Primary cell wall, IH: Internal hyphae of *Pythium debaryanum* and SP: simple pits.

Growth parameters

The performance of *P. roxburghii* seedlings inoculated with VAM fungus and infected with *P. debaryanum* treated with ASM or fungicide as well as uninoculated seedlings is shown in Fig. 2.

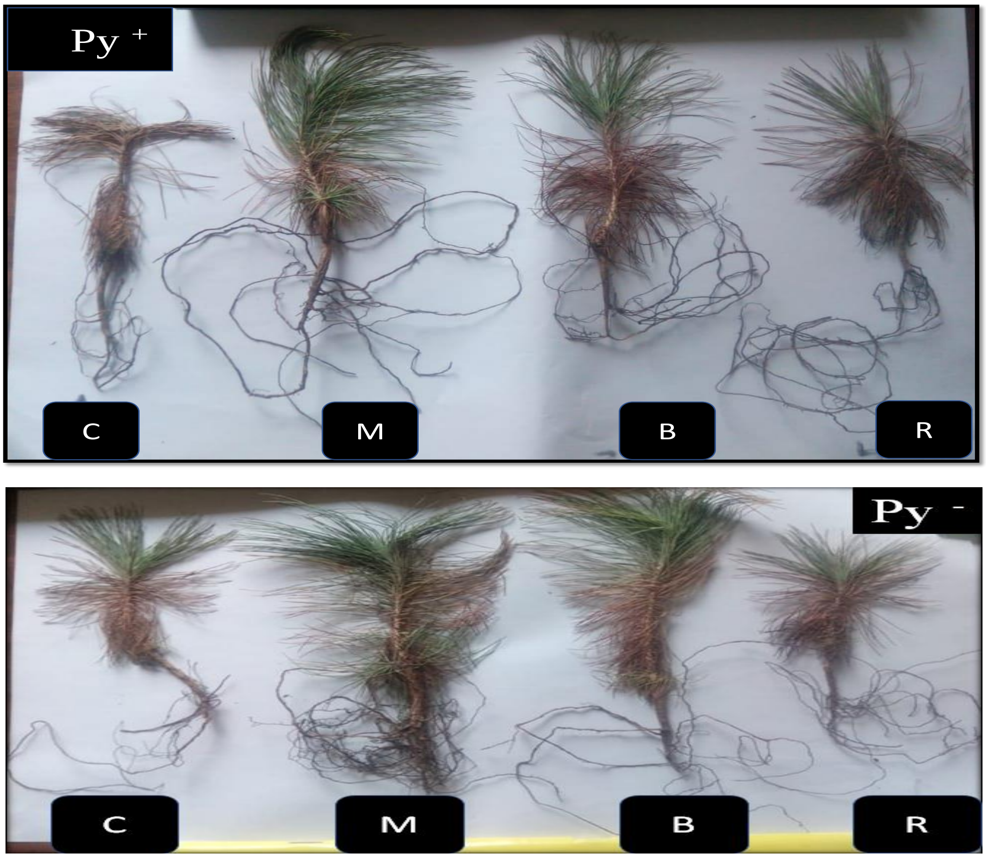

Figure 2: *Pinus roxburghii* seedlings inoculated and uninoculated with VAM fungus and infected with *P. debaryanum* which treated with ASM or fungicide. C: uninoculated seedlings (control); M: seedlings inoculated with mycorrhizas; B: seedlings treated with Boin; R: seedlings treated with Rhizolex

Shoot height

The uninfected with *P. roxburghii* seedlings displayed the lowest shoot height (SH) (16.03 and 15.76 cm) as compared with uninfected ones in both seasons, respectively (18.8 and 17.97 cm) as shown in (Table 3). There are significant differences owing to the effect of the main treatments. However, seedlings that inoculated with VAM displayed the highest SH in both seasons (23 and 22.5 cm), respectively, followed by those treated seedlings with Bion in both seasons (19.45 and 17.95 cm, respectively), yet there were non-significant differences between the untreated seedlings (control) and treated seedlings with Rhizolex in both seasons (Table 3).

As for the interaction between the two factors, the uninfected seedlings by *P. dybaryanum* and inoculated with VAM fungus displayed the highest SH in both seasons (24.1 and 24.1 cm respectively) (Table 3).

Live shoot ratio based on height (LSR)

The uninfected seedlings with *P. dybaryanum* displayed the highest LSR in the first and second season (0.7553 and 0.719), followed by the infected ones (0.5802 and 0.538) (Table 4). The inoculation with VAM has brought about the highest LSR in both seasons (0.7935 and 0.8000 for the first and second season, respectively), whereas the lowest value was in the control and the treated seedlings with Rhizolex in both seasons (Table 4).

The significant interaction between pathogen infection and the main treatments has manifested that the uninfected seedlings by pathogen and inoculated with VAM displayed the highest LSR in both seasons (0.858 and 0.8506, respectively), followed by the uninfected seedlings by pathogen and treated with Bion, in both seasons (0.8169 and 0.7688), respectively but there were non-significant differences among the impacts remaining treatments (Table 4).

Root length (RL)

The trend of the significance of data concerning RL is similar to that obtained in the case of LN (Table 5). However, there was a significant effect of the inoculation with pathogenic Oomycete on RL as uninoculated seedlings with fungus that displayed the highest RL in both seasons (12.15 and 11.55 cm, respectively). It is clear that the inoculation with VAM displayed the highest value of RL in both seasons (21 and 19.9 cm, respectively), while there is non-significant difference among the rest treatments (Table 5).

Finally, the significant dual interaction between the two studied factors manifested the positive impact of the inoculation with mycorrhiza, the inoculated seedlings with VAM and uninfected by pathogenic Oomycete displayed the highest RL in first and second season (23 and 21.3 cm, respectively), but there were non-significant differences among the impact of the remaining treatments. (Table 4).

Table 4: Live shoot height ratio based shoot length (LSR) (cm) of inoculated seedlings with VAM fungus and infected with *P. debaryanum* which treated with ASM or fungicide and uninoculated seedlings of *Pinus roxburghii* in the first and second season (2019-2020).

Pythium debaryanum (Py)	First season					Second season				
	Treatment					Treatment				
	C	VAM	Boin	Rhizolex	Means	C	VAM	Boin	Rhizolex	Means
Py+	0.00^{b}	0.72^{b}	0.66^{b}	0.64^{b}	0.58^{b}	0.00^{b}	0.74^{b}	0.61^{b}	0.61^{b}	0.53^{b}
Py-	0.64^{b}	0.85^{a}	0.81^{b}	0.60^{b}	0.75^{a}	0.57^{b}	0.85^{a}	0.76^{b}	0.56^{b}	0.71^{a}
TRT	0.41^{c}	0.79^{a}	0.74^{b}	0.62^{c}		0.32^{c}	0.80^{a}	0.70^{b}	0.59^{c}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Table 5: Root length (cm) of inoculated seedlings with *Glomus mosseae* fungus and infected with *P. debaryanum,* which treated with Bion or Rhizolex and un-inoculated seedlings of *Pinus roxburghii* in the first and second season (2019-2020).

Pythium debaryanum (Py)	First season					Second season				
	Treatment					Treatment				
	C	VAM	Boin	Rhizolex	Aver.	C	VAM	Boin	Rhizolex	Aver.
Py+	11.4^{b}	19^{b}	6.8^{b}	5.2^{b}	10.6^{b}	11.8^{b}	18.5	6.6b	5.1^{b}	10.5^{b}
Py-	8.4^{b}	23^{a}	7.6^{b}	9.6^{b}	12.15^{a}	8.1^{b}	21.3^{a}	7.4^{b}	9.4^{b}	11.55^{a}
TRT	9.9^{b}	21^{a}	7.2^{b}	7.4^{b}		9.95^{b}	19.9^{a}	7.00^{b}	7.25^{b}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus

Needle dry weight (NDW) (g)

Data in Table 6 showed that the un-inoculated seedlings with pathogenic Oomycete displayed the highest NDW value in both seasons (3.427 and 3.135 g), compared with the inoculated ones in both seasons (3.074 and 2.970 g). (Table 6). The inoculation with VAM has brought about the highest NDW in both seasons (3.964 and 4.109 g, respectively), However, there were no significant differences between inoculated and un-inoculated seedlings with VAM (control) in both seasons and treated ones with Rhizolex in NDW, since it is averaged 3.083 and 3.964 g, respectively (Table 6). The analysis of variance has revealed that all factors and their interactions have significant impacts, in terms of NDW.

Root dry weight (RDW)

Data in Table 7 revealed that there was a significant effect between treatments. However, the uninfected seedlings with Oomycete displayed the highest RDW in both seasons (3.021 and 2.945 g, respectively), while the RDW for the infected seedlings were (2.798 and 2.728 g, respectively) (Table 7). Concerning the effect of inoculation with symbiotic and chemical agents, there were significant differences between un-inoculated seedlings (control) and inoculated ones with the symbiotic agent. The inoculation with VAM exhibited the highest RDW (3.136 and 3.057g, respectively), yet the un-inoculated seedlings either infected with *P. debaryanum* or not displayed the lowest value of RDW in both seasons (2.562 and 2.497g, respectively) (Table 7). Upon significant interaction between inoculation with pathogenic Oomycete and inoculation with the symbiotic agent, it is clear that the inoculation with VAM in the case of the uninfected seedlings with pathogenic Oomycete induced the highest RDW values in the first and second seasons (3.524 and 3.435g, respectively (Table 7).

Total dry weight (TDW) (g).

Data in Table (8) indicated that, the uninfected seedlings with *P. debaryanum* gave the highest TDW in both seasons (5.219 and 6.219 g, respectively) compared with the infected ones which gave 4.288 and 5.288 g in the first and second seasons, respectively (Table 8). Concerning the effect of inoculation with symbiotic and chemical agents, there were significant differences between un-inoculated seedlings (control) and inoculated ones with symbiotic agents. In spite of infection with the pathogen, the inoculation with VAM exhibited the highest TDW in both seasons (5.863 and 6.863 g, respectively) (Table 8).

Based on the significant interaction between main factors and pathogens. The uninfected seedlings which inoculated with mycorrhiza had displayed the highest TDW in the first and second seasons (6.575 and 7.575 g respectively), but there were non-significant differences among the other treatments (Table 8).

Table 6: Needle dry weight (NDW) (g) of inoculated seedlings with *Glomus mosseae* fungus and infected with *P. debaryanum* which treated with Bion or Rhizolex and un-inoculated seedlings of *Pinus roxburghii* in the first and second season (2019-2020).

***Pythium debaryanum* (Py)**	**First season**					**Second season**				
	Treatment					**Treatment**				
	C	**VAM**	**Boin**	**Rhizolex**	**Aver.**	**C**	**VAM**	**Boin**	**Rhizolex**	**Aver.**
Py+	2.723^{d}	3.654^{b}	2.578^{d}	3.342^{c}	3.074^{b}	2.323^{c}	3.663^{b}	2.254^{d}	3.639^{b}	2.970^{b}
Py-	3.443^{b}	4.274^{a}	3.167^{c}	2.823^{d}	3.427^{a}	3.142^{b}	4.554^{a}	2.565^{c}	2.278^{c}	3.135^{a}
TRT	3.083^{b}	3.964^{a}	2.873^{b}	3.083^{b}		2.733^{b}	4.109^{a}	2.410^{b}	2.959^{b}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus

Table 7: Root dry weight (RDW) (g) of inoculated seedlings with *Glomus mosseae* fungus and infected with *P. debaryanum* which treated with Bion or Rhizolex and un-inoculated seedlings of *Pinus roxburghii* in the first and second season (2019-2020).

***Pythium debaryanum* (Py)**	**First season**					**Second season**				
	Treatment					**Treatment**				
	C	**VAM**	**Boin**	**Rhizolex**	**Aver.**	**C**	**VAM**	**Boin**	**Rhizolex**	**Aver.**
Py+	2.547^{d}	2.748^{c}	2.978^{b}	2.920^{c}	2.798^{b}	2.483^{d}	2.679^{c}	2.902^{b}	2.847^{c}	2.728^{b}
Py-	2.576^{d}	3.524^{a}	2.901^{c}	3.083^{b}	3.021^{a}	2.511^{d}	3.435^{a}	2.827^{b}	3.005^{b}	2.945^{a}
TRT	2.562^{c}	3.136^{a}	2.940^{b}	3.002^{b}		2.497^{c}	3.057^{a}	2.865^{b}	2.926^{b}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. Where: C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Table 8: Total dry weight (TDW) (g) of inoculated seedlings with *Glomus mosseae* fungus and infected with *P. debaryanum* which treated with Bion or Rhizolex and un-inoculated seedlings of *Pinus roxburghii* in the first and second season (2019-2020).

Pythium debaryanum (Py)	First season					Second season				
	Treatment					Treatment				
	C	VAM	Boin	Rhizolex	Aver.	C	VAM	Boin	Rhizolex	Aver.
Py+	3.350^{d}	5.150^{c}	4.850^{c}	3.800^{c}	4.288^{b}	4.350^{d}	6.150^{b}	5.850^{c}	4.800^{c}	5.288^{b}
Py-	5.075^{c}	6.575^{a}	5.675^{b}	3.550^{d}	5.219^{a}	6.075^{b}	7.575^{a}	6.675^{b}	4.550^{d}	6.219^{a}
TRT	4.213^{c}	5.863^{a}	5.263^{b}	3.675^{c}		5.213^{c}	6.863^{a}	6.263^{b}	4.675^{c}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Enzyme activities:

1-Polyphenol oxidase (PPO) activity

The un-inoculated seedlings with pathogenic fungus exhibited the highest average of PPO activity (0.465 and 0.546 Unit g^{-1}/ fresh wt., respectively) in both seasons (Table 9). There are significant differences owing to the effect of the inoculation with the symbiotic agents on PPO activity. Seedlings inoculated with VAM displayed the highest average of PPO activity (0.558 and 0.592 Unit g^{-1}/ fresh wt., respectively) in both seasons, followed by treated seedlings with Bion (0.505 and 0.579 Unit g^{-1}/ fresh wt., respectively) in both seasons (Table 9). Highlighting the significant interaction between the main factors, main treatments and the inoculation with pathogenic fungus indicated that the un-inoculated seedlings which inoculated with VAM had displayed the highest PPO activity (0.672 and 0.847 Unit g^{-1}/ fresh wt., respectively) in both seasons, followed by those treated with Bion (0.552and 0.672 Unit g^{-1}/ fresh wt., respectively) in both seasons (Table 9).

2-Peroxidase (POX) activity

In the case of the effect of inoculation with symbiotic and chemical agents, there were significant differences between un-inoculated seedlings (control) and inoculated ones with the previously mentioned symbiotic agents. VAM exhibited the highest peroxidase activity (0.642 and 0.716 Unit g^{-1}/1g fresh weight, respectively) in both seasons (Table 10). The analysis of the significant interaction between pathogenic Oomycete and the main treatments revealed that the un-inoculated seedlings with *p. debaryanum* which inoculated with VAM displayed the highest peroxidase activity (0.847 and 0.879 Unit g^{-1}/1g fresh weight, respectively) in both seasons, followed by those the un-inoculated with *p. debaryanum*, which applied with Bion (0.771 and 0.745 Unit g^{-1}/ 1g fresh weight, respectively) in both seasons. (Table 10).

Table 9: Polyphenol oxidase (PPO) (Unit g-1/ fresh wt.) (*in vivo*) during inoculation with symbiotic and pathogenic fungi in the first and second season.

Pythium	First season					Second season				
	Treatment					Treatment				
	C	VAM	Boin	Rhizolex	Aver.	C	VAM	Boin	Rhizolex	Aver.
Py+	0.224^{c}	0.443^{b}	0.458^{b}	0.242^{c}	0.317^{b}	0.256^{c}	0.336^{c}	0.486^{b}	0.290^{c}	0.342^{b}
Py-	0.344^{c}	0.672^{a}	0.552^{b}	0.292^{c}	0.465^{a}	0.395^{c}	0.847^{a}	0.672^{b}	0.271^{c}	0.546^{a}
TRT	0.284^{c}	0.558^{a}	0.505^{b}	0.267^{c}		0.326^{c}	0.592^{a}	0.579^{b}	0.281^{c}	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Table 10: Peroxidase activity (Unit g-1/ 1g freash weight) (*In vivo*) during inoculation with symbiotic and pathogenic fungi in both seasons.

Pythium (Py)	First season					Second season				
	Treatment					Treatment				
	C	VAM	Boin	Rhizolex	Aver.	C	VAM	Boin	Rhizolex	Aver.
Py+	0.234d	0.436c	0.464c	0.242d	0.344b	0.243c	0.553b	0.487bc	0.136d	0.355b
Py-	0.163d	0.847a	0.771b	0.292cd	0.518b	0.344c	0.879a	0.745b	0.142d	0.528a
TRT	0.199d	0.642a	0.618b	0.267c		0.294c	0.716a	0.616b	0.139c	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Table 11: Total phenolic compounds (µg/g) during inoculation with symbiotic and pathogenic fungi in both seasons.

Pythium	First season					Second season				
	Treatment				Aver.	Treatment				Aver.
	C	VAM	Boin	Rhizolex		C	VAM	Boin	Rhizolex	
Py+	5.65^c	27.63^a	20.98^a	8.22^c	15.62^a	6.74^c	29.74^a	20.36^a	8.15^c	16.25^a
Py-	1.82^d	16.51^b	12.93^b	4.41^d	8.92^b	2.41^d	17.68^b	13.63^b	6.44^d	10.04^b
TRT	3.74^d	22.07^a	16.96^b	6.32^c		4.58^d	23.71^a	17.00^b	7.30^c	

According to LSD at 0.05 level of probability, means with the same letter are not statistically different. C: un-inoculated seedlings (control); VAM: vesicular arbuscular mycorrhizal fungus.

Total phenolic compound content

A result of inoculation with symbiotic and chemical agents, there were significant differences between un-inoculated seedlings (control) and inoculated ones with the previously mentioned symbiotic agents. The inoculated seedlings with VAM exhibited the highest total phenolic (27.63 and 29.74 (µg/g), respectively) in both seasons (Table 11). The statistical analysis of the variance has revealed significant interaction between pathogenic Oomycete and the main treatments, The inoculated seedlings with *p. debaryanum*, which inoculated with VAM had displayed the highest peroxidase activity (27.63 and 29.74 (µg/g) respectively) in both seasons, followed by those inoculated with *P. debaryanum* which applied with Bion, (20.98 and 20.36 (µg/g) respectively) in the first and second season (Table 11).

DISCUSSION

Induced resistance has been studied in many horticultural and agricultural systems as it offers the possibility of broad-spectrum control of pests and diseases using the defense mechanisms of the plant itself had been paid (Vallad and Goodman, 2004; Walters, 2009). The synthetic chemical primer/activator, acibenzolar-S-methyl (trade name Actigard or Bion), has been used as a broad-spectrum protectant (Leadbeater and Staub, 2007). Moreover, the antifungal activity of ASM and its ability to reduce the growth of pathogens *in vitro* in certain fungi was observed (Faessel et al., 2008; Zine et al., 2016).

Results of the effect of elicitors on disease in the greenhouse revealed that all treatments significantly reduced the disease severity and increased fresh weights of pine seedlings compared to untreated control. These treatments improve plant health. Our results are in agreement with those reported by other researchers (Amini, 2015; Hofgaard et al., 2005; Małolepsza, 2006).

The majority of plant protection methods specifically target invasive pathogens. This type of behavior has generally been shown to reduce the effectiveness of pesticides by causing disease resistance (Hahn, 2014). The ecosystem and animal health are also negatively impacted by the usage of chemicals. In order to minimize the negative impacts of pesticide and pathogen adaptability, it is increasingly obvious that alternative and sustainable plant protection strategies are required (Pretty, 2018).

In this work, we found that the inoculation with mycorrhiza and treatment with ASM suppressed the growth of pathogens, and activated plant nature defense against disease. This result is in agreement with another researcher (Resende et al., 2002; Arabi et al., 2013; Ji et al., 2011; Resende et al., 2002).

The use of plant defense stimulators is one of the proposed alternate plant protection strategies, which is being investigated by researchers and farmers because they don' t directly target the pathogen and they provide a wide protection range. However, the plant defense stimulator's efficiency is controversial. While they can protect plants from pathogen infection under controlled conditions, their efficiency in the field is often unstable (Verly et al., 2020). Our results investigated the possibility that plant responses to plant defense stimulators could be affected by the inoculation with mycorrhiza when treated with ASM increased, these obtained results are in accordance with (Verly et al., 2020).

Our results indicated that ASM was effective against *Pythium debaryanum* in greenhouse experiments reduced disease severity and increased plant growth compared with the tested fungicide. The use of ASM is a potentially integrated pest management-based tactic to control damping off disease because its long-lasting efficacy allows the application of typical fungicides to be reduced. Our results are in agreement with what was found by other researchers (Elmer, 2006; Hage-Ahmed et al., 2019).

Since elicitors are still in their very early stages of application as a new control strategy for crop protection and pest management, the existing experiences are still based on laboratory trials rather than on extensive agricultural use.

Using elicitor therapies has at least been shown to have the several benefits, or they can be anticipated as protective agrochemicals, elicitors can be applied with current spraying technology (Cham et al., 2021). Additionally, elicitor treatments may be an alternative to genetically modified (GM) plants for better attraction of natural enemies of pest organisms on cultivated plants, reductions in damage from insects, fungi, pests, and herbivores and reductions in environmental hazards (Kappers et al., 2005; Poppy and Wilkinson, 2008).

The primary enzyme in the phenylpropanoid pathway, phenylalanine ammonia-lyase, is responsible for producing phenolic chemicals, which are linked to the expression of disease resistance (Treutter, 2006). Because Bion increased the activity of the enzyme phenylalanine ammonia-lyase in plants, Bion treatments may also result in higher levels of total phenolic content (Barilli et al., 2010; de Barros et al., 2019). In *Pinus* needles, Bion treatment has been shown to boost phenylalanine ammonia-lyase activity, which in turn increases the total phenolic content.

The browning reaction of the tissues is caused by the oxidation of phenolic substances in plant cells, which is thought to be a sign of pathogen infiltration (Pratyusha, 2022). Furthermore, polyphenoloxidase stimulates the metabolization of these phenolic chemicals into more dangerous forms (Chrzanowski et al., 2003). In addition, phenolic component concentration increased following the treatments compared to the untreated control. In this regard, phenols undergo oxidation to produce more toxic quinones or semi-quinones, which are important antibacterial agents (Farkas and Kiraaly, 1962).

When compared to the non-mycorrhizal, non-pathogen-treated control, total phenol levels increased in this study in all treatments, but they were at acme rate when plants as a result of the antagonistic impact both the mycorrhizal fungus on the pathogenic one.

CONCLUSIONS

Bion treatments and VAM inoculations improved plant growth and yield, the accumulation of some antimicrobial substances like phenolic compounds, and the activity of enzymes involved in defense. Plant resistance to *P. debaryanum* infection was also increased. In order to find out an alternative fungicide, such therapy may be utilized as a component of integrated disease control for field crops. Further research on the economics of using this PGPF (mycorrhiza), which can be utilized commercially on a wide scale conditions, is emphasized.

AUTHOR CONTRIBUTIONS

Marwa G. S. Frahat and Mohamed R. A. Shehata both contributed equally to the methodology, writing, and reviewing of the article.

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الملخص العربي

تعزيز مقاومة شتلات الصنوبر ضد فطر البيثيوم باستخدام فطريات الميكوريزا الداخليه وماده اسيبنزولار– أس – ميثيل

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أجريت هذه الدراسة لتقييم التأثيرات المثبطة المحتملة للفطريات الفطرية الحويصلية المفصلية (VAM)، و acibenzolar-s-methyl (Bion®) ، ومبيد الفطريات Rhizolex® 50WP ضد فطر *Pythium debaryanum*. أظهرت النتائج أن التلقيح بـــ VAM كان قادرًا على تقليل نمو المسبب المرضي بشكل ملحوظ. أظهرت المعاملة بالـــ Bion فعالية مضادة للفطريات عالية ضد فطر*Pythium debaryanum* . أظهرت شتلات *Pinus roxburghii* التي تم تلقيحها بـــ VAM أعلى معايير النمو من حيث ارتفاع المجموع الخضري والوزن الجاف للجذر والوزن الجاف الإجمالي في كلا الموسمين، تليها الشتلات المعالجة بالبيون في كلا الموسمين. كشفت الاختبارات الأنزيمية أن الميكوريزا والبيون كانا قادرين على تعزيز مستويات أنشطة الإنزيم المرتبطة بمقاومة الأمراض بشكل ملحوظ، بما في ذلك البيروكسيديز والبولي فينول أوكسيديز، والتي كانت مرتفعة بشكل واضح مقارنة بالشتلات غير المعاملة (الكنترول). أظهرت الميكورايزا تأثيرا أعلى على زيادة أنشطة الإنزيمات االمرتبطة بمقاومة الأمراض مقارنة بالبيون. اظهرت النتائج ارتفاع نسبة الفيونولات الكلية في جميع المعاملات بالمقارنة مع بالشتلات غير الملقحة بالميكوريزا وغير المعداه بالمسبب المرضي، ولكنها كانت أعلى عندما تم تلقيح النباتات بكل من الفطريات الميكوريزا والممرض. توصي هذه الدراسة بالتلقيح بالميكوريزا الحويصلية الشجرية VAM لزيادة الأداء الإنتاجي لشتلات *Pinus roxburghii* والتحكم في نمو فطر *Pythium debaryanum*