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Isolation of Potential Antagonistic Rhizosphere Fungi against *Alternaria alternata* from Organic Carrot Productions

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ABSTRACT

Rhizospheric fungi can have serve as biological agents against plant pathogens *Alternaria alternata*, which infects carrot plantss. Rhizospheric fungi are more abundant in organic lands. This study aims to isolate fungi from organic carrot's rhizosphere that are able to suppress *A. alternata*'s growth and determine isolate rhizospheric fungi ability to inhibit *A. Alternata* to identify potential biological control agents against *A. alternata*. This research was conducted at the Laboratory of Biotechnology and Basic Biology, Diponegoro University, between January–June 2023. Research methods include survey location, soil sampling, rhizospheric fungi isolation, pathogenic fungi isolation, creating growth curve, pathogenicity tests, antagonism tests, and fungi identification. This study used a complete randomized design (CRD) and was analyzed using ANOVA at the 5% level of significance and Duncan's hoc-posttest. Fungi isolation from organic carrot plant's rhizosphere resulted in 13 isolates consisting of 6 genera: *Penicillium, Aspergillus, Paecilomyces, Myrothecium, Trichoderma,* and *Simplicillium*. The antagonism test showed that 8 antagonistic isolates, including the genus *Penicillium* (WO1, WO6, WO10), *Paecilomyces* (WO5), *Myrothecium* (WO7), *Trichoderma* (WO9), and *Aspergillus* (WO11, WO13), where WO1, WO6, WO10, and WO11 have a medium percentage of inhibition (44.12-57.84%), while WO5, WO7, WO9, and WO13 have a high percentage of inhibition (64.29-76.06%). The eight isolates showed antagonism mechanisms, including antibiosis (WO1, WO5, WO6, WO7, WO10, and WO11) and parasitism (WO9 and WO13).

Keywords*: Alternaria* leaf blight; antagonist fungi; antagonism test; carrot plant rhizosphere; organic soil

INTRODUCTION

Soil microorganisms, including fungi, are one of the components of soil biodiversity that play a vital role in the soil ecosystem. According to Noviyanti *et al.* (2022), fungus can act as a decomposer and play a vital role in soil fertility by providing nutrients for plants. Among other factors that influence the existence of fungi is the way land is managed. Organic land production systems that use organic materials and are based on environmentally sustainable practices. Organic land is known to have a higher abundance of soil microorganisms than conventional land (Das & Dkhar, 2011; Nurbailis *et al.*, 2014).

Carrot (*Daucus carota* L.) is one of many highly consumed vegetables because of its high nutritional content. According to Bender *et al.* (2020), organic carrots also have several advantages, such as higher market-demand for organic products, low nitrate content, high vitamin C and beta-carotene content, as well as lower chemical residue levels. Based on Soesanto *et al.* (2013), pathogens are challenges in plant production because they can reduce yield production. An example of carrot pathogen is *Alternaria alternata*, which causes leaf and plant blight (Soyal *et al.*, 2018; Gayithri *et al.*, 2021; Yadav *et al.*, 2021). Control of *A. alternata* has been largely done with the use of synthetic fungicides. The use of synthetic fungicides cause harm to the environment and humans. Thus, environmental friendly disease management are needed, for example, using antagonistic fungi (Muksin *et al.*, 2013). Rhizospheric fungi that are abundant in organic soils are opportunities to identify potential biocontrol agents against pathogens that attack carrot plants, inclduing *A. alternata*. The objectives of this study include examining the antagonism ability of fungi isolated from the rhizosphere of organic carrot plants against *A. alternata*, examining the macroscopic and microscopic characteristics of fungi from the rhizosphere of organic carrot, and determine types of fungi with potential to suppress the growth of *A. alternata*.

MATERIALS AND METHODS

Research Location. The soil sampling from organic land was done at "*Argo Ayuning Tani*" farmer group in Senden Village, Selo District, Boyolali Regency (7.4849918, 110.4716418). The research was carried out at Biotechnology and Basic Biology Laboratory, Biology Department, Faculty of Science and Mathematics, Diponegoro University.

Soil Sampling. Soil was collected from ares directly in contact with healthy carrot roots from 3 plant samples, were randomly selected, and combined into one as a composite sample (Purwantisari *et al.*, 2016). The sample was stored in a cool box (18 x 28 x 10 cm) and brought to the laboratory for further isolation (Amaria *et al.*, 2013).

Isolation and Purification of Rhizospheric Fungi. A total of 10 g of soil sample was dissolved in 90 mL of sterile distilled water and homogenized using a shaker at 120 rpm for 15 minutes at room temperature ($25-30$ °C). One mililiter was transferred to a test, which contained 9 mL of distilled water, homogenized to obtain 10^{-1} dilution. Dilutions were repeated until 10^{-5} dilution was obtained. One mililiter of the suspension resulting from $10^{-1}-10^{-5}$ dilution was taken and transferred aseptically to a petri dish containing Potato Dextrose Agar (PDA) medium (Himedia, India) added with chloramphenicol. Fungi was incubated for

3−5 days at room temperature (Yulia *et al.*, 2022). Fungi grown from a 10^{-5} dilution were purified based on the appearance of the colonies by transferring each colony to a new sterile PDA.

Preparation of Growth Curves. Growth of each purified isolate grown using the single dot method was observed. Colony diameter was measured every day until the 15th day. Then a growth curve is made from these results (Hasanah, 2018).

Isolation of Pathogenic Fungi. Symptomatic carrot plants were sampled and infected tissues were directly plated. Samples were washed thoroughly using water, then cut into 1×1 cm pieces using a scalpel. The sample surface was then sterilized by immersion in a 70% alcohol solution for 30 seconds and NaOCl 1% for 1 minute, then rinsed using sterile distilled water. Furthermore, the sample pieces were dried on sterile tissue paper and planted on sterile PDA medium that had been treated with chloramphenicol. Then, the culture was incubated for 2–5 days at room temperature (Asrul *et al.*, 2021). The growing fungi were purified for use in the pathogenicity test, and identification of the pathogenic fungi was carried out based on their macroscopic and microscopic characteristics.

Pathogenicity Test. Healthy carrot tubers were washed thoroughly using water and cut into ± 5 mm thick pieces (Tülek & Dolar, 2015). The tuber pieces were then sterilized by immersion in 70% alcohol for 1 minute and 1% NaOCl for 1 minute, then rinsed using sterile distilled water three times (Fadhilah *et al.*, 2014). The tuber pieces were placed on a sterile petri dish that has been given a piece of sterile filter paper that is moistened with sterile distilled water. The tuber sections were then injured and inoculated with 5 mm mycelial-plugs of rejuvenated pathogenic cultures. Control was inoculated using agar on tuber pieces (Tülek & Dolar, 2015). The cultures were then incubated for 7 days, and symptoms were observed.

Antagonism Test. Rhizospheric and pathogenic fungi at the same growth phase (log phase) were inoculated on Petridishes (9 cm of diameter) containing PDA medium at a distance of 3 cm. Incubated for 7 days at room temperature and radius of the pathogen in the treatment and control were measured to then calculate the percentage of inhibition (%) using the the following equation (1) (Živković *et al.*, 2010):

$$P = \frac{(r1 - r2)}{r1} x 100\% \qquad (1)$$

P: inhibition percentage (%)

r1: pathogen colony radius in control

r2: pathogen colony radius in treatment

Observation of antibiosis can be seen from the presences of a clear zone between two fungi coloy (Nurbailis *et al.*, 2014). While antagonism test that showed high inhibition, contact with hyphae, and the invasion by rhizosphere fungi, were further observed for their parasitism ability using the slide culture method to observed penetration, coiling, or lysis of pathogenic hypha under a microscope (Nurbailis *et al.*, 2014).

Identification. This was done by observing the macroscopic and microscopic characteristics of the isolates. Observed characteristics were matched with several identification books, i.e. *Pictorial Atlas of Soil and Seed Fungi Morphologies of Cultured Fungi and Key to Species* (2nd Edition) by Watanabe (2002), *Illustrated Genera of Imperfect Fungi* (4th Edition) by Barnett and Hunter (1998), *Illustrated Genera of Imperfect Fungi* (2nd Edition) by Barnett (1960), and *Food and Indoor Fungi* by Samson *et al.* (2010).

Data Analysis. The antagonism test was carried out using a completely randomized design (CRD) with three replications for each treatment. The number of treatments carried out was same as isolated rhizosphere fungi. Data were then analyzed for variance using a 5% ANOVA and Duncan's further test.

RESULTS AND DISCUSSION

Rhizospheric fungi isolation from organic carrot plants resulted in 13 types of fungi (Table 1). Pathogenic fungi isolated from symptomatic carrot plants was identified as *A. alternata* (Figure 1). This fungus showed leaf blight on isolated carrot plants. According to Soyal *et al.* (2018), Chrapačienė *et al.* (2021), Gayithri *et al.* (2021), Shebl *et al.* (2021), and Yadav *et al.* (2021), all confirmed that *A. alternata* causes leaf blight in carrots.

Based on the results of the *in vitro* pathogenicity test, pathogenic fugal isolates caused black rot on the tuber pieces. Based on previous research by Shebl *et al*. (2021) and Tülek & Dolar (2015), an *in vitro* pathogenicity test can be conducted on carrots. Although *A. alternata* is a leaf blight pathogen, it still showed root rot symptoms. This is in accordance with Shebl *et al.* (2021), who also conducted a pathogenicity test on carrot tubers and resulted in tuber rot caused the growing leaves to have lesions and then wither. This was also stated by Chrapačienė *et al.* (2021) and Yadav *et al.* (2021), *A. alternata* can also cause root rot in carrots.

Fungal growth consists of several stages; this is in accordance with Prayitno and Hidayati (2017), who stated that fungal growth consists of an adaptation phase, an

exponential phase, a stationary phase, and a death/autolysis/cell damage phase. The results of measuring the diameter of each isolate of rhizosphere fungi and pathogenic fungi show different growth rates (Figure 2). Speed of fungal growth results based on daily diameter increase, successively from the fastest resulted in: WO9, WO13, PAA, WO4, WO8, WO3, WO7, WO1, WO5, WO12, WO2, WO11, WO6, and WO10. Highest growth rates for was shown by WO9 (*Trichoderma* sp.) and WO13 (*Aspergillus* sp. 5) demonstraing growth rates faster than *A. alternata* by 4-folds while other isolates grew slower than *A. alternata*. Fungi with high growth rates and antagonistic mechanisms such as antibiotics and parasitism are potential biocontrol agents. According to Halwiyah *et al.* (2019), fungi with high growth rates are able to absorb nutrients and compete with pathogens, but slow growing fungi can still be potential biocontrol agents when they show antagonistic mechanisms such as antibiotics and/or parasitism.

The antagonistic test of rhizosphere fungi against *A. alternata* consisted of 13 treatments (Figure 3). Analysis of variance (ANOVA) showed that the inhibition percentage of antagonistic fungi significantly affected *A. alternata* in vitro at the 7th day (Table 2). Inhibition percentage of isolate WO1 (*Penicillium* sp. 1) was not significantly different from WO2 (*Penicillium* sp. 2) and WO3 (*Aspergillus* sp. 1), where all three had an inhibition percentage of 48.57%. The inhibition percentage of isolate WO4 (*Aspergillus* sp. 2) was 57.14% and not significantly different from isolate WO8 (*Aspergillus* sp. 3) that was 57.14%, isolate WO10 (*Penicillium* sp. 4) was 57.84%, and isolate WO12 (*Simplicillium* sp.) was 55.88%. The inhibition percentage of isolate WO5 (*Paecilomyces* sp.) was 64.29% and not significantly different from isolate WO7 (*Myrothecium* sp.) which was 65.20%. The inhibition percentage of isolate WO9 (*Trichoderma* sp.) was not significantly different from isolate WO13 (*Aspergillus* sp. 5), both of which were 76.06%. While the inhibition percentage of the WO6 isolate (*Penicillium* sp. 3) was 44.12%, and WO11 isolate (*Aspergillus* sp. 4) was 50.00%, both were significantly different from other rhizosphere fungi isolates.

According to Yulia *et al.* (2022), inhibition ability by antagonistic fungi are categorized into low (0–29%), moderate (30–59%), and high (60–100%). Isolates with a high inhibition percentage, included WO5 (*Paecilomyces* sp.) was 64.29%, WO7 (*Myrothecium* sp.) was 65.20%, WO9 (*Trichoderma* sp.), and WO13 (*Aspergillus* sp.5) were 76.06%. Meanwhile other isolates have a moderate percentage of inhibition, including WO1 (*Penicillium* sp. 1), WO2 (*Penicillium* sp. 2), WO3 (*Aspergillus* sp. 1) were 48.57%; WO4 (*Aspergillus* sp. 2) and WO8 (*Aspergillus* sp. 3) were 57.14%; WO6 (*Penicillium* sp. 3) was 44.12%; WO10

(*Penicillium* sp. 4) was 57.84%; WO11 (*Aspergillus* sp. 4) was 50.00%; and WO12 (*Simplicillium* sp.) was 55.88%.

Antagonism tests against *A. alternata* pathogenic fungi showed several different mechanisms (Table 3). All isolates showed a competition mechanism, but among the 13 isolates, there were isolates that showed an antibiosis mechanism, including WO1 (*Penicillium* sp. 1), WO5 (*Paecilomyces* sp.), WO6 (*Penicillium* sp. 3), WO7 (*Myrothecium* sp.), WO10 (*Penicillium* sp. 4), and WO11 (*Aspergillus* sp. 4). This was indicated by the clear zone between the antagonistic isolate and the pathogen isolate indicating that the six isolates produce secondary metabolites that inhibit the growth of the pathogen. Based on Khokhar *et al.* (2011) and Gupta and Rodriguez-Couto (2017) *Penicillium* sp. can produce compounds that act as antifungals, namely formamidine, uvidin, thymol, camazulene, altenusin, dehydroaltenusin, and fatty acids. Moreno-Gavira *et al.* (2020) and Moreno-Gavira *et al.* (2021) stated that *Paecilomyces* sp. can produce secondary metabolites as antimicrobials, including antifungals, 6-decyl salicylic acid, eicosenoic acid, ascofuranone, botulin, paecilaminol, paeciloxazine, etc. Based on Elkhateeb and Daba (2019) and Xiong *et al.* (2021), *Myrothecium* sp. can produce compounds in the form of myrothins AF, which act as antifungals against several pathogenic fungi that cause root rot; verrucarins and roridins, which act as mycotoxins; and triterpene glycosides, which inhibit pathogenic fungi in vitro. Whereas WO11 (*Aspergillus* sp. 4), which belongs to the subgenus *Polypaecilum*, mentioned by Pitt and Hocking (2022) that *Polypaecilum* does not produce harmful mycotoxins. Also, research conducted by Sabariah (2002) found that *Polypaecilum* can produce metabolites that inhibit aflatoxin production by *A. parasiticus* and *A. flavus*; these metabolites include -1,4 glycosides, L-amino acids, and lysyl or argynyl residues.

While WO9 (*Trichoderma* sp.) and WO13 (*Aspergillus* sp. 5) were capable of invading or growing against pathogenic fungi in dual culture (Figure 3), WO9 and WO13 isolates have the highest inhibition activity and were capable of invading *A. alternata*. So, we observed the mechanism of parasitism with the slide culture method and showed the results under the microscope in Figure 4.

The mechanism of parasitism by WO9 (*Trichoderma* sp.) isolates in the form of implantation, followed by coiling (a1) where the antagonist hypha envelopes the pathogen hypha, then the penetration (a2) of antagonistic hypha penetrates into the lumen of the hypha cell pathogen, as well as the enzyme degradation of the cell hypha fungus pathogen by antagonistic fungus (a3). The same is true of WO13 (*Aspergillus* sp.5) which shows an implantation that is followed by enlargement (b1), then penetration (b2) by antagonistic

fungal hypha into pathogenic cell hypha lumen with the help of cell wall degradation enzymes, as well as the enlightenment (b3) of the pathogen hypha by the antagonist fungus. This is in line with Amaresan *et al.* (2020), which stated that mycoparasitism is a complex mechanism consisting of several stages, including identification and chemotropism, implantation and depilation, secretion of extra-cellular enzymes, penetration of cell walls by hypha, and degradation of the host hypha cell wall.

The process of penetration and smoothing is due to the presence of cell wall degradation enzymes. *Trichoderma* and *Aspergillus* are known to have cell wall-degradation enzymes that play a role in parasitism. According to Herrera-Estrella (2014), *Trichoderma* sp. can produce the enzymes chitinase, glucanase, and protease involved in mycoparasitism. As Hu *et al.* (2014) mentioned, *Aspergillus* sp. could be mycoparasite with the production of chitinase and glucanase. WO9 and WO13 isolates can both produce cell wall degradation enzymes, especially chitinase, but they resist or do not experience self-degradation because they have genes that can encode cell wall protective proteins. It is consistent with Hu *et al.* (2014) and Gruber & Seidl-Seiboth (2012), which mention that chitinase can be produced by some species of *Aspergillus* spp., which has a protein-coding gene that protects the hydrophobic cell wall, similar to that found in *Trichoderma* spp.

CONCLUSION

Rhizospheric fungi isolated from organic carrot plants resultted in 13 species that have different characteristics. The fungi found belonged to six different genera: *Penicillium* sp., *Aspergillus* sp., *Paecilomyces* sp., *Myrothecium* sp., *Trichoderma* sp., and *Simplicillium* sp. The antagonistic abilities were showed by isolate WO1 (*Penicillium* sp. 1), WO5 (*Paecilomyces* sp.), WO6 (*Penicillium* sp. 3), WO7 (*Myrothecium* sp.), WO9 (*Trichoderma* sp.), WO10 (*Penicillium* sp. 4), WO11 (*Aspergillus* sp.4), and WO13 (*Aspergillus* sp. 5). WO1, WO6, WO10, and WO11 have moderate inhibition (44.12-57.84%) with antibiotic mechanism, whereas WO5, WO7, WO9, and WO13 isolates have high inhibitory percentages (64.29-76.06%) and show antibiosis mechanisms by WO5 and WO7, as well as parasitism by WO9 and WO13. The eight isolates could potentially be used as biocontrol agents if isolate a later proven or certified as non-pathogenic to host plants before application to plants.

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APPENDIX

Table

Table 1. Identification of rhizosphere fungi isolated from organic plants

No	Isolate code	Fungi genus
1	WO1	*Penicillium* sp. 1
2	WO2	*Penicillium* sp. 2
3	WO3	*Aspergillus* sp. 1
4	WO4	*Aspergillus* sp. 2
5	WO5	*Paecilomyces* sp.
6	WO6	*Penicillium* sp. 3
7	WO7	*Myrothecium* sp.
8	WO8	*Aspergillus* sp. 3
9	WO9	*Trichoderma* sp.
10	WO10	*Penicillium* sp. 4
11	WO11	*Aspergillus* sp. 4
12	WO12	*Simplicillium* sp.
13	WO13	*Aspergillus* sp. 5

Table 2. Antagonism test of rhizosphere fungi against *Alternaria alternata* on the 7th day

Treatment/isolate	Inhibition Percentage (%)
WO1	48.57 ab
WO2	48.57 ab
WO3	48.57 ab
WO4	57.14 c
WO5	64.29 d
WO6	44.12 a
WO7	65.20 d
WO8	57.14 c
WO9	76.06 e
WO10	57.84 c
WO11	50.00 b
WO12	55.88 c
WO13	76.06 e

Note: Means followed by the same lowercase alphabet in the same column were not significantly different based on Duncan's multiple range test at the level of 5 %.

Table 3. Antagonism mechanisms of rhizosphere fungi isolates *on Alternaria alternata*

Treatment	Mechanism		
	Competition	Antibiosis	Parasitism
WO1 X PAA	+	+	-
WO2 X PAA	+	-	-
WO3 X PAA	+	-	-
WO4 X PAA	+	-	-
WO5 X PAA	+	+	-
WO6 X PAA	+	+	-
WO7 X PAA	+	+	-
WO8 X PAA	+	-	-
WO9 X PAA	+	-	+
WO10 X PAA	+	+	-
WO11 X PAA	+	+	-
WO12 X PAA	+	-	-
WO13 X PAA	+	-	+

Note: (+) indicates a mechanism; (-) indicates no mechanism; PAA (Pathogen *Alternaria alternata)*

Charts
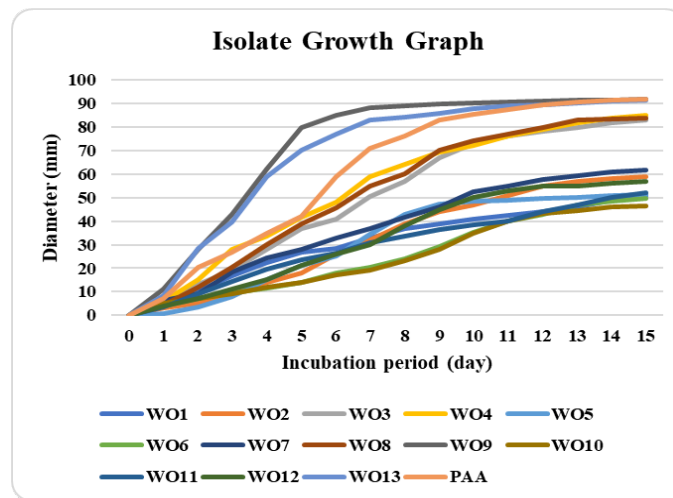
Figure 2. Growth curve of carrot rhizosphere fungi from organic fields (WO1 to WO13) and pathogenic fungi (PAA) in PDA.
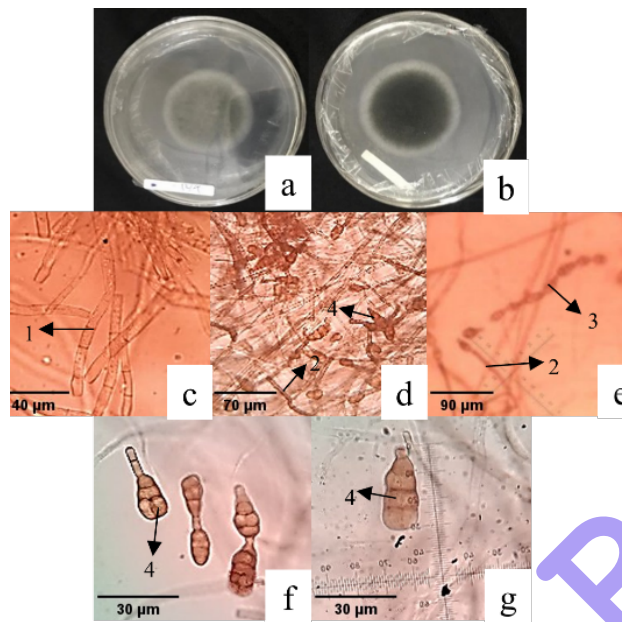
Images

Figure 1. Characteristics of *Alternaria alternata.* a. Upper colony, b. Reverse colony, c, d, e, f, g. are microscopic features(1: hyphae insulated; 2: conidiophores; 3: series of; 4: conidia).
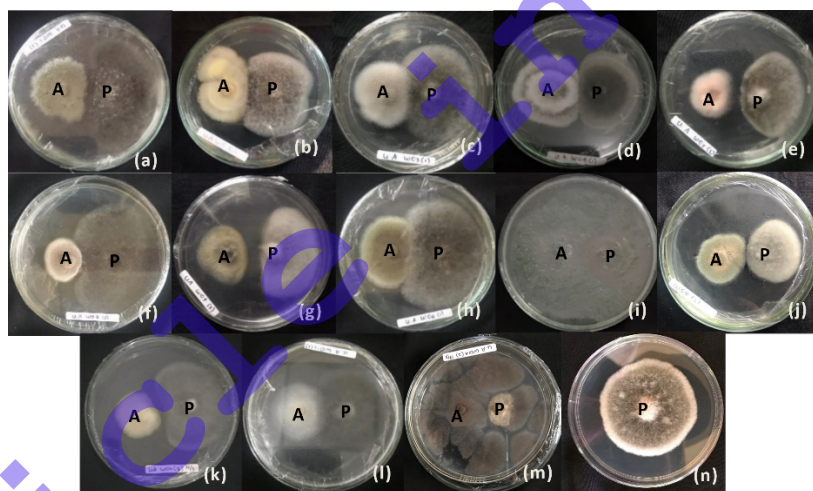

Figure 3. Antagonism test of rhizosphere fungi against *Alternaria alternata* on the 7th day. (note: (a):WO1; (b): WO2; (c):WO3; (d):WO4; (e):WO5; (f):WO6; (g):WO7; (h):WO8 ; (i):WO9; (j):WO10; (k):WO11; (l):WO12; (m):WO13; (n) control; A: Antagonistic fungi; P: Pathogenic fungi)
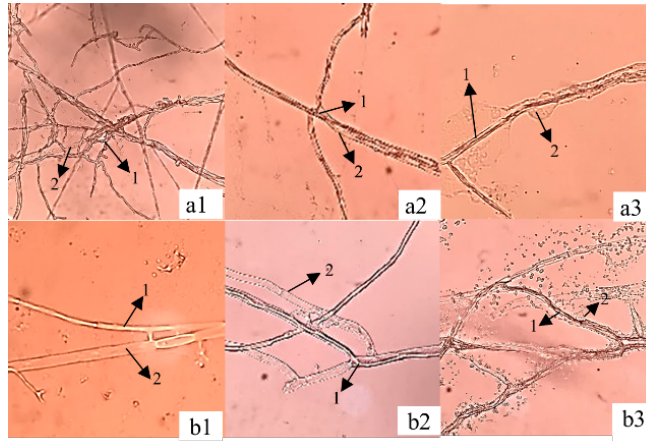
Figure 4. Mycoparasitism by WO9 (a1. attachment and coiling; a2. penetration; a3. lysis), and WO13 (b1. attachment; b2. penetration; b3. lysis); (Remarks: 1: antagonistic fungal hyphae; 2: pathogenic fungal hyphae)