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Biotechnical control of tar spot (*Rhytisma acerinum*) disease on velvet maple (*Acer velutinum* Boiss) *in vitro*

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ABSTRACT: Several different fungi can cause tar leaf spot diseases in maple trees, including three fungi of the genus *Rhytisma*. *Rhytisma* a*cerinum* (Pers.) Fries is an ascomycete that forms black stromata known as tar spot on the adaxial surface of the leaves of *Acer* species. The tar spot (*R. acerinum*) disease has been increasing in incidence and severity in maples of Hyrcanian forests, northern Iran, in recent years. One of the best ways to manage infestations by *R. acerinum* is through adequate biotechnical techniques. The isolation of fungal spore colonies was evaluated using different dosages of Oxywet 10% (50, 100, 200, 500 µl), Gentamicin 5% (100, 200, 400; 1,000 µl), and Amoxicillin antibiotics 20% (25, 50, 100, 250 µl) in 100 ml of distilled water in each treatment. All possible combinations of single doses were applied using light and dark treatments. In light conditions, it appears that the Oxywet (200 µl) had the significant effect on controlling *R. acerinum*. Reduced fungal growth, coefficient and inhibition of fungal growth were observed in the light treatment. The other antibiotics (Gentamicin, Amoxicillin) were not so effective in controlling this pathogen. Results of spore germination showed a significant difference between all treatments. All treatments were tested in pure cultures in the laboratory only. The results obtained cannot be expected of the same effectiveness in open field trials.

Keywords: antibiotic; fungus

The genus *Acer* (commonly known as maple) is comprised of 150 widely distributed species in the temperate zone, ranging from North America, Europe, and Asia to North Africa. Maples are highly valuable for a variety of purposes, such as lumber and veneer, ornamental uses, food and shelter for wildlife and watershed protection (Farhadi et al. 2013). *Acer velutinum* Boiss (velvet maple) is one of the most valuable native species in northern Iran, which is widely distributed in the Alborz mountain range from 200 to 2,000 m in elevation. Under favourable conditions, velvet maple reaches over 40 m in height and diameters exceeding 150 cm. It comprises nearly 8% of the growing stock in this area and provides nearly 2% of domestic wood demand (Farhadi et al. 2007). The fast growth of this species has drawn the attention of forest managers as one of the key species used in reforestation of deforested or degraded lands. However, seed dormancy has hampered attempts to raise seedlings in tree nurseries for large-scale planting (Difazio et al. 1988; Yousef-Zadeh et al. 2007).

Rhytisma acerinum is a plant pathogen that commonly affects maples in late summer and autumn, causing a problem known as tar spot (Hudler 1998). Tar spot infection remains localized to the chlorotic areas on the leaves creating mostly a cosmetic issue, rather than an economically detrimental disease. *Rhytisma acerinum*, a biotrophic ascomycete parasite, locally infects the leaves of maple trees (Weber, Webster 2002). Symptoms first appear as inconspicuous, pale green to yellow areas on the leaves. As the fungus grows within the leaf, these areas develop into distinctive, slightly raised, shiny, tar-like, black spots on the leaves. The size of the spot depends upon the fungal species;

spots can be irregular and up to 1.3 cm in diameter (*R. acerinum*) or can appear as tiny, pinpoint dots (*Rhytisma punctatum*). Significant premature autumn coloration and defoliation can occur, especially when the infection is heavy, as is often the situation in Norway maple (Hudler, Banik 1987; Hsiang et al. 2008).

Rhytisma acerinum can occur in many tree species, although it most commonly infects species of the genus *Acer*. The large tar spot (*R. acerinum*) most commonly infects Norway maple (*Acer platanoides*), silver maple (*Acer saccharinum*), sycamore maple (*Acer pseudoplatanus*) and recently velvet maple (*A. velutinum*). The small tar spot (*R. punctatum*) occurs in madrone (*Arbutus menziesii*), yellow poplar (*Liriodendron tulipifera*) and willow (*Salix*) (Sinclair et al. 1987; Tainter, Baker 1996; Hsiang, Tian 2007).

Tar spots contain the teleomorph of *R. acerinum*. In the spring, needle-shaped ascospores are released from overwintering apothecia in fallen leaf debris (Weber, Webster 2002; Hsiang et al. 2009). The wind disseminates the spores, which have a sticky coating allowing them to attach to new healthy leaves. Once on the leaves, the spores germinate and penetrate through the stoma. The subsequent infection causes chlorosis of the leaves in localized yellow spots. As the season continues into summer, apothecia begin to form, giving rise to brown-black leaf lesions that resemble spots of tar. Leaves retain their yellow border during the initial chlorosis. Apothecia overwinter in the fallen plant debris, releasing spores when the temperature warms again. The use of adequate sanitation techniques provides one of the best methods of managing the pathogen. Because the fungus overwinters in diseased leaf debris, removing the debris in autumn can help reduce the occurrence of the disease. In certain severe cases, fungicides can also assist with control. Copper-based fungicides sprayed in early spring when leaves are budding and twice more throughout the season also helps to reduce the incidence of the disease (Hsiang et al. 2009).

The objectives of this study were to determine (*i*) appropriate biotechnical method that can be used to control *R. acerinum*, (*ii*) suitable concentrations of selected antibiotics, and (*iii*) to analyze individual and combined effects of biotechnical antibiotic on fungal growth, area under fungi growth curve, called the AUFGC coefficient, and inhibition of fungal growth. Here it is essential to state that all this was tested in pure cultures in the lab only. The results obtained cannot be expected of the same effectiveness in open field trials.

MATERIAL AND METHODS

Sterilization and isolation of *R. acerinum*. For isolation and sterilization of *R. acerinum* colonies, infected leaves from tar spot samples were collected from the Shast Kalate forest, Gorgan, northern Iran (36°43'–36°48'N and 54°21'–54°24'E) (Etminan et al. 2011). The surfaces of the leaves were sterilized before separation of the fungal spots. Samples of spots on leaves were isolated, cut into approximately 5 × 5 mm sections, and the surfaces were sterilized with 0.05% NaOCl and EtOH for 2 min (Hudler, Banik 1987), rinsed with 96% alcohol for one min and then washed with distilled water under laboratory conditions. Leaf cultures on 4% (w/v) Potato-Dextrose-Agar (PDA))Pinkerton, Strobel 1976 (media were incubated at 24 ± 1°C and, after 21 days, the isolation of *R. acerinum* was performed. Cultures of *R. acerinum* were exposed to three antibiotics: Oxywet, Gentamicin and Amoxicillin. The selected treatments were Oxywet (50, 100, 200, 500 µl), Gentamicin 5% (100, 200, 400; 1,000 µl) and Amoxicillin 20% (25, 50, 100, 250 µl) in 100 ml distilled water. Each was prepared in conditions of both light and darkness. The growth rates of the *R. acerinum* colony and mycelium were studied to test the effect of each antibiotic and radial growth was measured for a week.

In the next step for the preparation of a spore suspension, a liquid potato-dextrose agar medium was used. Two circles of the fungus from five mycelia at the edge of fungi colonies were transferred to media in laminar air flow. The media were placed on shakers set at 70 rpm. After 14 days, the media were transferred from sterilized Whatman filter paper onto a vacuum hopper for separating the mycelium. Samples were centrifuged about three times, for 10 min at 10,000 rpm. Spores in suspension were counted with a haemocytometer for achieving 2×10^2 spores per ml. To evaluate the effects on spore germination, the three antibiotics tested here were integrated with PDA media. One µl of the suspension was distributed at the media level. Rates of inhibition of spore germination were determined by counting germinated spores after 7 days for each treatment. The spores obtained were exposed under conditions of light and darkness in media containing the three antibiotics: Oxywet, Gentamicin and Amoxicillin.

Treatments used were 10% Oxywet at four concentrations (50, 100, 200, 500 µl), 20% Amoxicillin at four concentrations (25, 50, 100, 250 µl) and 5% Gentamicin at four concentrations (100, 200, 400; 1,000 µl) in four replications, with all tests com-

pleted under conditions of both light and darkness. Equation (1) was used to calculate the AUFGC on various days:

$$AUFGC = \frac{\Sigma_{i+1}^{n}(X_{i+1} + X_i)}{2}(t_{i+1} - t) \quad (1)$$

where:

X_i – observed colony size,

t – time (days),

n – number of observations.

Statistical analysis. This study was conducted as a two-factor factorial experiment with a completely randomized design. Means were compared using Duncan's new multiple range tests with the results considered significant at $P < 0.01$.

RESULTS

Results showed that 200 µl of Oxywet antibiotic in 100 ml distilled water prevented the fungal colony growth of the maple tar spot disease pathogen (*R. acerinum*) and caused the degradation and growth of fewer fungal mycelium filaments of *Rhytisma acerinum* (Fig. 1).

The effect of light and antibiotic on the AUFGC factor did not show any significant differences ($P < 0.01$) but the type of antibiotic used resulted in a significant difference in AUFGC ($P < 0.05$) (Table 1). The mean comparison of different antibiotics on AUFGC showed that among used antibiotics, 200 µl of Oxywet had a minimal effect on AUFGC (Fig. 2).

In dark conditions, Oxywet antibiotic with concentrations of 50, 100, 500 µl had the maximum impact on the AUFGC coefficient and the 200 µl Oxywet concentrations had the minimum impact on the AUFGC coefficient (Fig. 3a) in light conditions, Oxywet in concentrations of 50, 100 and 500 µl and control treatment had a significant impact on the AUFGC coefficient and Oxywet with concentrations of 200 µl had the smallest impact on the AUFGC coefficient (Fig. 3b).

The effect of the antibiotic in light and darkness on the mycelial growth of the fungus was not significantly different between treatments. The effect of the antibiotic on the mycelial growth of the fungus was significantly different (Table 1). Based on Fig. 4, the mean comparison of various treatments on the mycelium growth of maple tar spot fungus shows that Oxywet with 50, 100 and 500 µl and amoxicillin with 250 µl had the maximum effect.

Table 1. Analysis of variance

Treatment	df	*P*
AUFGC coefficient		
Light	1	0.22^{ns}
Antibiotic	12	0.015*
Light × antibiotic	12	0.20^{ns}
Growth of fungus mycelium		
Light	1	0.96^{ns}
Antibiotic	12	0.001**
Light × antibiotic	12	0.98^{ns}
Spore germination of *Rhytisma acerinum*		
Light	1	0.001**
Antibiotic	12	0.002**
Light × antibiotic	12	0.005**

*$P < 0.05$, **$P < 0.01$ – significant differences between the treatments; ^{ns} not significant

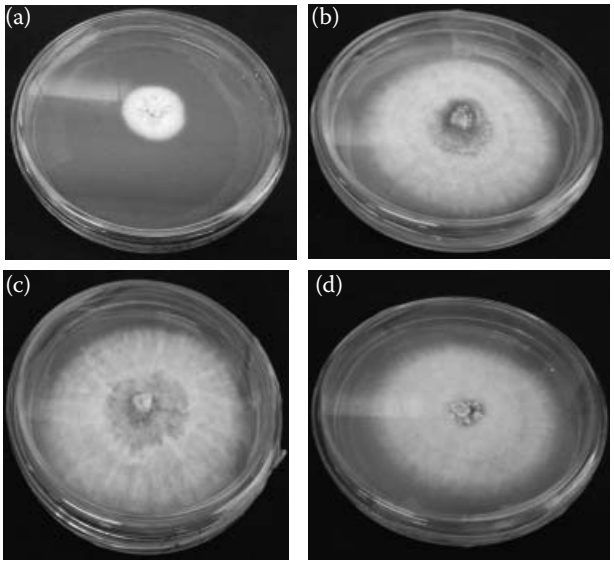

Fig. 1. The fungus *Rhytisma acerinum* in different treatments: (a) Oxywet 200 µl, (b) Gentamicin, (c) Amoxicillin and (d) control treatment

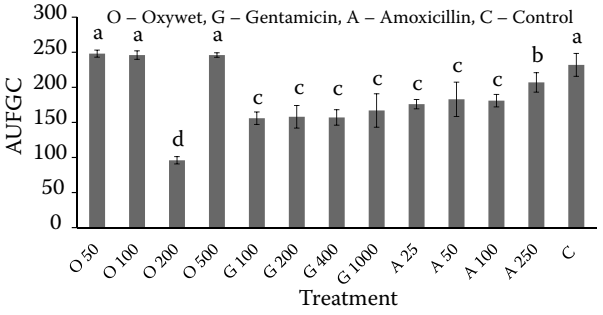

Fig. 2. Mean (± SE) comparison of AUFGC coefficient in different treatments. The same letters indicate mean values are not significantly different

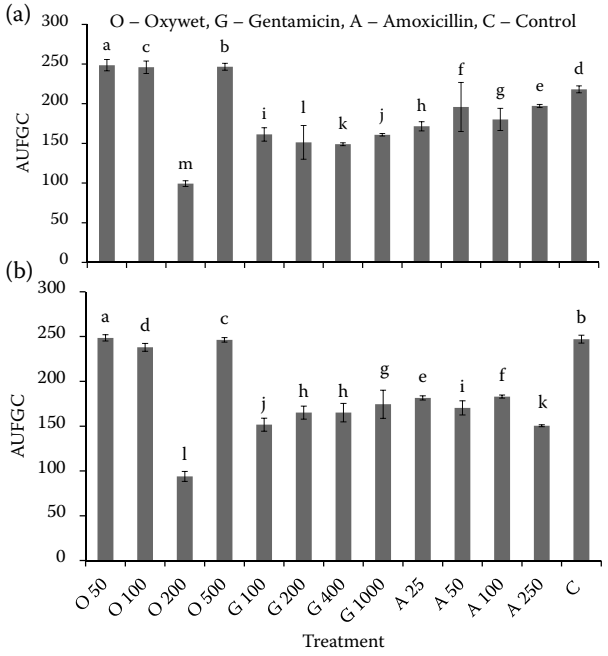

Fig. 3. AUFGC mean (±1 SE) in different treatments in terms of dark (a) and light (b). The same letters indicate mean values that are not significantly different

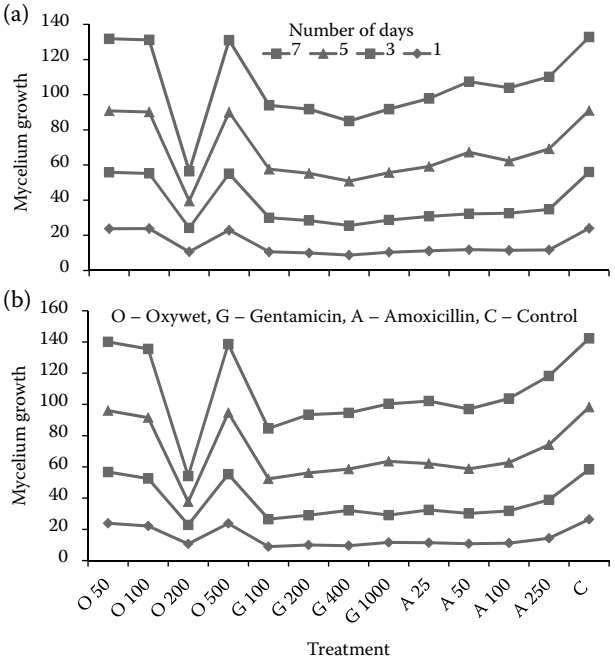

Fig. 5. The fungal mycelium growth of *Rhytisma acerinum* in dark (a) and light (b) conditions at different times after culture

Fig. 5a shows that Oxywet 200 µl was the least effective in preventing the fungus mycelial growth in the absence of light. Of the other media containing different antibiotics, Amoxicillin (25 and 250 µl) and Oxywet (50 and 250 µl) in 100 ml of distilled water had the greatest impact on the mycelium growth in darkness. Fig. 5b indicates that Oxywet of 200 µl in light conditions had the smallest effect on the fungal mycelium growth. The other Oxywet concentrations (50, 100 and 500 µl) can stop the fungal mycelium growth in light conditions and it was the most effective antibiotic.

The analysis of antibiotic and light effects on spore germination showed that the effects of antibiotic and light during spore germination were significantly different (Table 1). The results of the mean comparison on spore germination showed the highest germination rate in the control and in the treatment of germination spores were released at roughly the same level and other antibiotics the spore germination could have been used to control (Fig. 6).

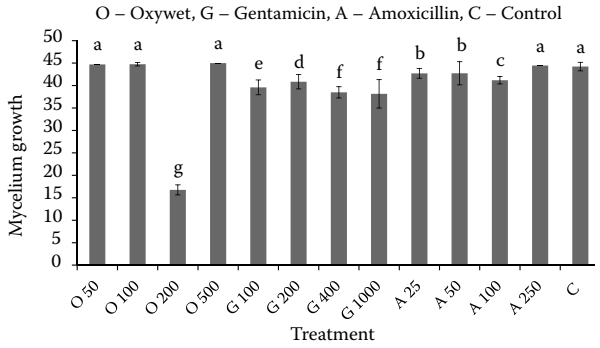

Fig. 4. Mean (±1 SE) comparison of the fungal mycelium growth in different treatments. The same letters indicate mean values that are not significantly different

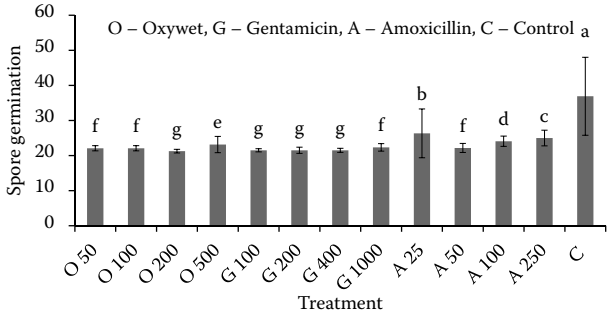

Fig. 6. Mean (±1 SE) comparison of different treatments on the spore germination of *Rhytisma acerinum*

Among the antibiotics used here, Oxywet 200 µl had the greatest impact on deterrence in dark conditions and Oxywet antibiotic concentrations of 50, 100 and 500 µl showed a minimum effect on the inhibition of the fungal mycelium growth (Fig. 7a). As Fig. 7b shows, the Oxywet antibiotic had the effective impact on growth inhibition in light conditions at 200 µl and Oxywet had the smallest effect with concentrations of 50, 100 and 500 µl. The other antibiotics initially inhibited the growth of the mycelium but over time, the ability to inhibit the mycelium growth was lost. Overall, 200 µl of Oxywet inhibited the uniform mycelium growth.

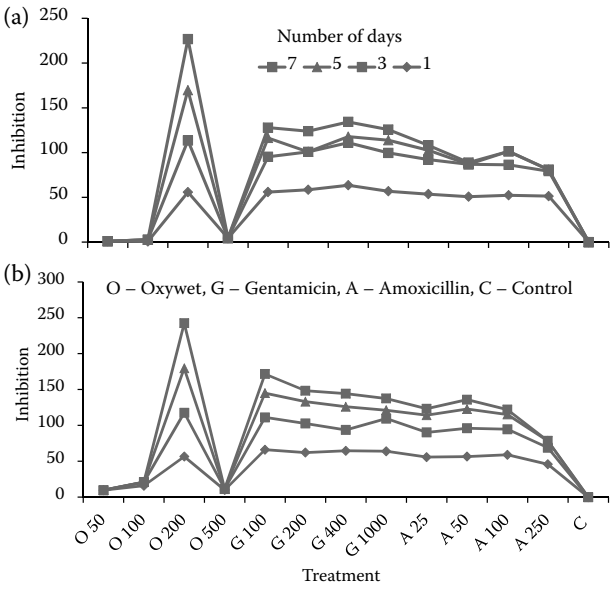

Fig. 7. Inhibition growth curve of the *Rhytisma acerinum* fungus in dark (a) and light (b) conditions at different time after culture

DISCUSSION

Several maple species are growing in the Hyrcanian region; of these the following velvet and Cappadocian maples are widely distributed in different parts of the Caspian forests (Sagheb-Talebi et al. 2014). Velvet maple is one of the fast-growing and productive tree species in the Caspian region that accounts for 5.8% of the standing volume and 2.7% of the stem number in the Hyrcanian forests of Iran (Rasaneh et al. 2001). Some individuals of velvet maple can reach large dimensions: 50 m in height and more than 2 m in diameter. It is a light demanding species and can be found as individual trees or in small groups from the plateau up to 2,000 m a.s.l. in different forest communities.

The most important pathogen on this tree species is *R. acerinum*. In general, tar spot diseases appear on maple leaves in early autumn, causing a reduction in wood and seed production. Finding an appropriate way to control this disease is very important. The results of this study demonstrate the effects of three antibiotics, Oxywet, Gentamicin and amoxicillin in different concentrations, on controlling the tar spot disease. Oxywet with a 200 µl concentration was effective in that it had a minimal impact on the AUFGC coefficient and Oxywet at 50, 100 and 500 µl concentrations had the greatest impact on the AUFGC coefficient. The effect of light and light antibiotic on the AUFGC coefficient and growth was not significant but the effect of antibiotic on the AUFGC coefficient and growth was significant by using Oxywet 200 µl antibiotic colonies; the mycelium was damaged and the other concentrations of this antibiotic (50, 100 and 500 µl) were not effective in controlling this disease. Based on the results, in terms of the presence and absence of light, Oxywet 200 µl showed the greatest impact on the amount of the minimum inhibitory effect on the fungus mycelium growth and Oxywet (50, 100 and 500 µl). Amoxicillin (25 and 50 µl) in terms of light and darkness had the minimum inhibitory effect and the maximum effect on mycelium effect as mycelium growth in addition to the control that showed the highest growth in media containing 50, 100 and 500 µl of the antibiotic. The presence or absence of light had a small effect on inhibiting the mycelium growth. In this study, spore germination in light, antibiotic and reciprocal effect of antibiotic and light resulted in significant differences in spore germination. In controlling spore germination in contrast to mycelium growth and fungal colonies, only Oxywet 200 µl was able to control the mycelium growth; the other antibiotics controlled the disease according to comparison. Light or darkness conditions were effective in spore germination as in light conditions; spores of the tar maple spot fungus (*R. acerinum*) were more able to germinate rather than to look for light.

CONCLUSION

Acer velutinum Boiss (velvet maple) is one of the most popular trees for plantations and restoration of different forest stands in the Caspian region, and is widely produced in nurseries. Tar spot caused by ascomycete fungi is problematic for maples in some parts of the world and can even cause health disorders in some vertebrates. This paper is concerned with the biotechnical control of the pathogen *in vitro*. There has been relatively little research done on tar spot in northern Iran.

This was not a successful attempt; this was a basic experiment for checking the effects of certain antibiotics against *Rhytisma mycelia* in the laboratory with quite promising results. And to conclude that they can be effective in controlling maple tar spot is too early. For that, additional tests would be necessary!

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