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Original Article

In vitro evaluation of plant essential oils against *Alternaria alternata* causing fruit rot of grapes

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Abstract

Alternaria fruit rot (AFR) caused by *Alternaria alternata* is a serious threat to grape market values in Pakistan during post-harvest stages, at the time of handling, storage transportation and distribution respectively. The objective of this study is to determine the anti-fungal activities of plant essential oils against *Alternaria alternata* causing fruit rot of grapes. For this purpose, three selected plant essential oils (EOs) *viz.* Kikar (*Acacia karoo*), Moringa (*Moringa oleifera*) and Sukh chain (*Pongamia pinnata*) essential oils at 200, 400 and 600 ppm concentrations were investigated by using different methods under *in vitro* condition against previously isolated culture of *A. alternata* designated as (Isolate ID. AA4PL1) on grape bunches. Results showed that Plant EO of Sukh chain at all concentrations showed significant result to inhibit the mycelial growth (89.4, 92 and 96.2 percent) in contact assay method as well as 96.2, 97 and 98.2 % growth inhibition regarding fungal culture transfer (FCT) experiment while, in case of well diffusion method 32%, 41% and 48% growth inhibition was recorded at 7^{th} day of incubation followed by Moringa EO and Kikar EO as compared to control that showed 0 % growth inhibition was measured. Moreover, results related to spore germination assay revealed that Sukh chain essential oil at 200, 400 and 600 ppm showed significant inhibition of germ tube length of *A. alternata* (140.8 µm, 77.5 µm and 34.1 µm) as compared to control in which germ tube length was recorded 250 µm respectively. It was concluded that Sukh chain EO has a great potential to inhibit the growth of *A. alternata* and can be further used as a strong antifungal agent against this pathogen under *in vivo* condition.

Keywords: Grapes, *Alternaria alternata*, Fruit rot, Management, Plant essential oils

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Introduction

Grapes (*Vitis vinifera* L.) are widely cultivated, economically important and highly nutritious fruit throughout the world (Ali et al., 2010). Grapes are also familiar as a "Queen of fruits" due to good source of multi-vitamins such as A & C as well as it contains a lot of bioactive compounds such as anthocyanins, carotenoids and some important antioxidants which has an important role to enhance the immune system (Rathi and Rajput, 2014). In Pakistan, grapes are mostly cultivated for fresh consumptions covered area of about 14 thousand Hectare with an annual production of 57 thousand tons. Besides its nutritional and medicinal values grape is one of the perishable fruit, having limited shelf life up to 3 to 4 days at ambient temperature. The maximum perishability of this fruit during handling, storage and marketing is due to susceptibility toward numerous post-harvest fungal diseases like Mucor rot (MR) (Ghuffar et al., 2018a), Botrytis bunch rot (Javed et al., 2017) *Penicillium* rot (PR) (Ghuffar et al., 2018b) and especially Alternaria fruit rot (AFR) associated with *Alternaria alternata* is responsible for weight loss, colour changes, softening of grape berries, increases the market losses up to 50 % and eventually has badly impact on economy (Valero et al., 2006). In addition, *Alternaria* genus produce mycotoxin such as tenuazonic acid (TA), alternariol monomethyl ether (AME) alternariol (AOH) these mycotoxins are carcinogenic for human health and physically deteriorate the whole bunches of grapes (Logrieco et al., 2009). For the control of Alternaria rot, Farmers spray synthetic fungicides on small fruits. However, these fungicides have some residual effect on berries skin which may lead to development of resistant fungi, oncogenic risk, handling hazards, and threats to the environment (Daferera et al., 2003). Therefore, many restrictions regarding application of chemical fungicides on small fruits are banned in many countries of the world (Arroyo et al., 2007; Tzortzakis, 2007). Nowadays, researchers have keen interest to provide some safer alternatives which have non-hazardous effects on environment as a replacement of chemical fungicides for farmers. They successfully found some biological methods, such as use of plant essential oils (EOs) are an exciting alternative. These Plant essential oils are volatile compounds, broad spectrum, anti- fungal activity, eco-friendly and more acceptable to the public (Wang *et al*., 2007). Keeping in all the view, the present study was conducted to find out the efficacy of different plant essential oils against *Alternaria alternata* causing fruit rot of grapes under *in vitro* condition.

Material and Methods

Collection of pathogenic fungal culture

The culture of *Alternaria alternata,* previously isolated from infected grape bunches of Perlette cv. designated as isolate AA4PL1 was from mycology lab, Department of Plant Pathology, PMAS-Arid Agriculture University Rawalpindi (AAUR) with Genebank submission ID. Alt 05, Accession No. MF785102 (Ghuffar et al., 2018c) respectively. Stock culture of fungal isolate was preserved at 4 °C in glass vials by using silica gel technique. Preserved fungal culture of *Alternaria alternata* was re-cultured by transferring beads onto freshly prepared Potato Dextrose Agar (PDA) in Petri dishes and incubated for 7 days at 25 °C. After revival, culture was purified and placed in incubator for seven days at 25°C using PDA media and finally used for further management trials under *in vitro* conditions.

In vitro screening of *Aternaria alteranta* by using plant essential oils (EOs) Extraction of plant essential oils (EOs) through Soxhlet's apparatus

Matured Leaves of sukh chain (*Pongamia pinnata*), moringa (*Moringa oleifera*) and small branches of kikar (*Acacia karoo*) were taken from Dajal Rajanpur and horticultural research station of PMAS- UAAR. These botanical materials were first dried under shadow, ground well in grinder machine and subjected for extraction process through Soxhlet's apparatus followed by (Sahin et al., 2003). Finally, extracted Plant essential oils (EOs) was in a clean glass vials and stored in refrigerator at 4°C until further tested

In vitro contact assay

To find out the efficacy of different plant essential oils on mycelial growth of *Alternaria alternata* poisoned food technique was used. For this purpose, 50 ml of prepared Potato Dextrose Agar media were kept in 100 mL conical flasks, sterilized for 20 min and kept under sterilized hood to cool up to 60°C then plant EOs were added to each flasks and shacked gently to prepare PDA media

containing 200, 400 and 600 ppm of concentrations. 9 cm Petri plates were poured with PDA containing known concentrations of plant EOs. 5 mm plug of 7 days old culture of *A. alternata* were kept in the center of each Petri plate whereas, in control sets PDA free of any essential oils were used. After that Petri dishes were incubated at ± 25 °C for 7 days and finally, mycelial inhibition concentration percentage (MIC) was recorded by using the following formula (Dauria et al., 2005):

$$\text{MIC } \% = \frac{c-t}{c} \times 100$$

where c means (diameter of control), t (diameter of treatment). The Analysis was replicated 3 times.

Fungal culture transfer Experiment

Transfer experiment was done to check the viability of the fungal culture which utilized in contact assay. For this purpose, from seven days old contact assay fungal plates, 6 mm plugs of fungal culture were shifted in a new plate. No essential oils (EOs) were used at this stage, later plates were incubated at ±25 °C for 7 days. Mycelial growth of the pathogen was measured by using formula described earlier. The data was recorded after 7th day of incubation with three replications (Feng and Zheng, 2007).

Well diffusion technique

Agar well diffusion is reliable technique used to screen out the anti-fungal activity of different plants essential oils. According to this method, two wells were made aseptically with a sterile cork borer at equidistant from each other and one well was filled by *Alternaria alternata* inoculum (10^7 spores/mL) while second with plant essential oils at defined concentrations of 200, 400 and 600 ppm respectively. Furthermore, Agar plates were incubated at ±25 °C. The plant (EOs) were diffused in the agar medium which inhibited the growth of the *A. alternata.* Linear growth inhibition was measured after 7 days and mycelial growth of pathogen was calculated by using the formula as same as previously described *in vitro* contact assay experiment.

Spore germination assay

The experiment was conducted in Potato Dextrose Broth (PDB) to find out the impact of most effective essential oil on germ tube length of *Alternaria alternata*. For this experiment, glass tubes were used having 10 ml space and poured with 5 ml PDB in each glass tube. After that aliquots (100 ul) having spore suspensions with concentration of (10^7 spores/ml) of *A. alternata* was added to each tube. Glass tubes were incubated at ±28 °C for 20 h on a rotatory shaker at 200 round per minutes (rpm). The germ tube length was observed after 20 hours with the help of microscope. Three Readings were taken for formula followed by (Feng & Zheng, 2007).

Statistical analysis

Statistical analysis of all experiments were conducted in triplicate and data were expressed as mean ± standard deviation after analyzed via CRD two factorial design (statistics 10.0 v). The statistical significance was set at a confidence level of $p < 0.05$.

Results

***In vitro* contact assay**

After the application of three selected plant essential oils under *in vitro* condition by using contact assay method. Data recorded after 7 days revealed that Sukh chain EO was most effective to inhibit the mycelial fungal growth 89.4, 92 and 96.2 percent at all applied concentrations (200, 400 and 600 ppm) followed by Moringa EO 58.9, 65.7, 68.3% while Kikar EO showed least efficacy 46.4%, 55.3% and 59.2% respectively. Whereas, in control set none of mycelial inhibition % was recorded as shown as in Figure 1

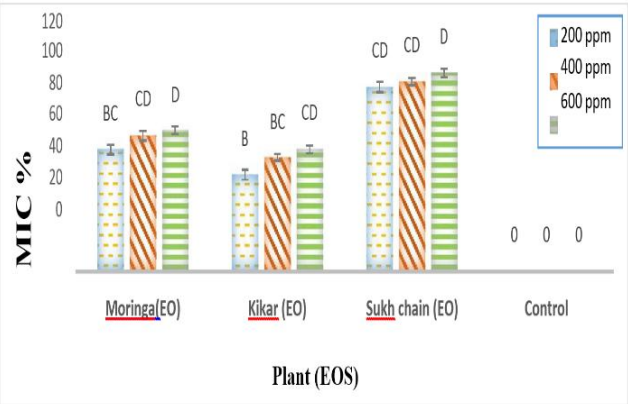

Figure-1: Efficacy of Plant essential oils at different concentrations after 7 days of incubation against *Alternaria alternata*

Fungal culture transfer experiment

A Similar result was found regarding in transfer experiment on 7th day demonstrated that Sukh chain essential oil at concentrations 200, 400 and 600 ppm showed significant result 96.2, 97 and 98.2 percent followed by Moringa oil 62.5,
66.7 and 71.5 % value while Kikar oil showed minimum effectiveness (52.5, 58.2 and 63.2 %) as compared to control where 0 % growth inhibition was recorded (Figure 2).

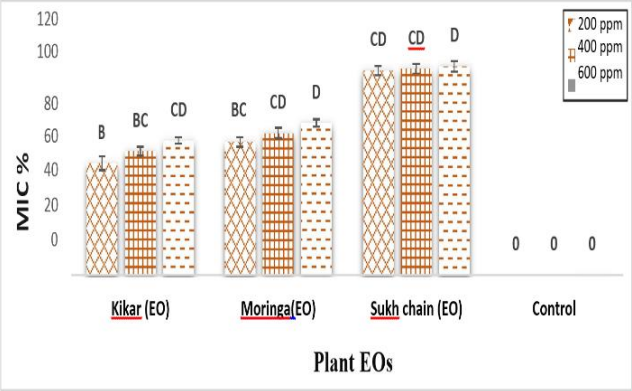

Figure-2: Effectiveness of Plant (EOs) on growth inhibition of *Alternaria alternate* on 7th day through transfer experiment

Well diffusion method

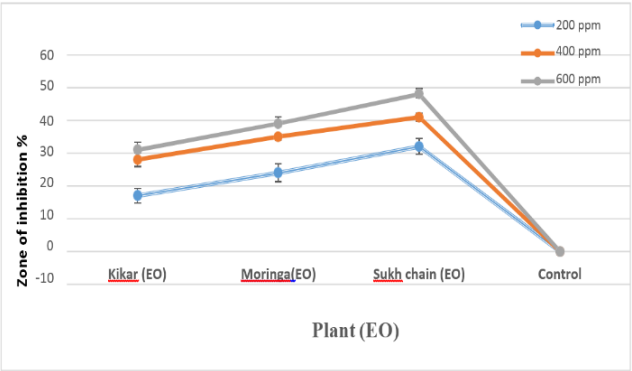

Figure-3: Effect of plant essential oils applied at three (200 ppm, 400 ppm and, 600 ppm) concentrations on growth inhibition zone of *Alternaria alternata* through well diffusion technique

From three selected plant essential oils Sukh chain EO was applied at pre-defined three doses of 200 ppm, 400 ppm and 600 ppm per well through well diffusion technique produce 32%, 41% and 48% growth inhibition zone percent respectively at 7th day of incubation after adding extract and fungal spore suspension in their respective wells while, Moringa EO showed the inhibition zone of pathogen at 600 ppm and 400 ppm doses after Sukh chain EO by expressing 39 % and 35 % growth inhibition respectively while 200 ppm dose showed 24% zone of inhibition at 7th day of incubation. In case of Kikar EO expressed growth inhibition of 31% at dose of 600 ppm while 28% as well as 17% at doses of 400 ppm and 200ppm per well in PDA media as compared to control which showed 0% growth inhibition (Figure 3). Fungal growth was recorded at 7 day of incubation.

Spore germination assay

After conducting *in vitro* contact assay and well diffusion experiments to determine the effectiveness of plant essential oils against *Alternaria alternata.* Sukh chain (EO) showed maximum growth inhibition % and used for further in spore germination assay experiment.

Length of germ tube

Sukh chain inhibited the germ tube length of *Alternaria alternata* on 7 days at concentrations (200, 400 and 600 ppm) but had some variations in terms of length measurement. At 200 ppm concentration germ tube length was recorded 140.8 µm under microscope observations followed by 77.5 µm length measured at 400 ppm while minimum length of 34.1 µm at 600 ppm concentration as compared to control germ tube length was recorded maximum 250 µm respectively (Figure 4).

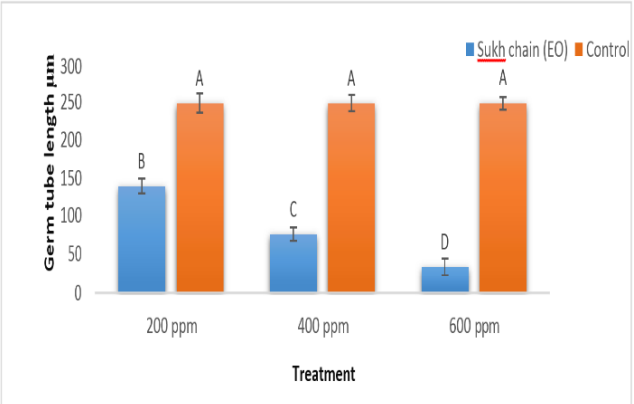

Figure-4: Germ tube length (µm) of *Alternaria alternata* on 7th day at different concentrations (200, 400, 600ppm) of sukh chain oil

Discussion

In last few years, use of synthetic fungicides are the primary means of controlling post-harvest pathogens but have some limitations due to resistance to fungicides among fungal pathogens and high development cost of synthetic chemicals. Therefore, researchers has successfully introduced alternatives in the replacement of chemical fungicides especially plant derived products as disease control agents due to less toxic effects, eco-friendly and wide public acceptance (Seema et al., 2011). In few years, interest on plant EO has been increased for the control of post-harvest fungal pathogens due to environmental friendly nature. In this study, we examined the anti- microbial activities of some plant essential oils (Kikar, Moringa and Sukh chain) against *Alternaria alternata* causing fungal fruit rot and demonstrated that Sukh chain EO among all tested plants at different concentrations had considerable effect on the growth rate and spore germination respectively. Similar findings were also reported by Das et al. (2016) revealed that *Pongamia pinnata* (Sukh chain) essential oils exhibited its maximum zone of inhibition (12.98 mm) against *A. alternata* due to presence of some chemical compounds which are anti-fungal in nature *viz.* saponin, phenolic, triterpenes and flavonoid respectively. Our findings regarding mycelial growth inhibition are related with results demonstrated by Tripathi et al. (2013). According to their results, concentrations of plant essential oils are directly effect on fungal growth of pathogens. Higher concentrations of essential oils can inhibit maximum fungal growth. Recently, some studies have been conducted by several scientists on determination of anti-fungal activity of plant essential oils against fungal pathogens including *Alternaria alternata* (Satish et al., 2007; Jamil et al., 2007; Anwar and Rashid, 2007). The mechanism of plant essential oils involve, inhibition of hyphal growth, interruption in nutrient uptake, disruption of mitochondrial structure and eventually disorganization of fungal pathogens discussed by (Patel and Jasrai, 2011). Numerous plant essential oils like *P. pinnata* (Sukh chain), *Vachellia nilotica*, *Thymus vulgare* and *Moringa oleifera* have been found to be effective fungitoxic agents against several plant fungal pathogens reported by (Siripornvisal and Ngamchawee, 2010). Our result are in agreement with the findings of Feng and Zheng, (2007) who used cassia oil at 500ppm concentration for controlling the germ tube length of *Alternaria alternata*, The findings in this study confirmed that Sukh chain essential oil might be used as natural fungicides.

Conclusion

The results obtained in the current study illustrated that Sukh chain oil has anti-fungal activity against *Alternaria alternata* under *in vitro* conditions. So, being less harmful and cheaper remedy, it can be used for controlling the *A. alternata* under field conditions.

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Contribution of Authors

Sajid A: Performed the experiments and manuscript write up
Irshad G: Planned and designed research experiments
Naz F: Planned and designed research experiments
Ghuffar S: Planned and designed research experiments
Hassan I: Planned and designed research experiments
Mahmood N: Data collection and analysis
Rani K: Data collection and analysis
Manzoor MF: Article write up and final approval
Meesam A: Article write up and final approval
Hamzah AM: Article write up and final approval
Karamt MZ: Performed the experiments