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Full Length Research Paper

Assessment of the antifungal activity of *Nicotiana glauca* Graham aqueous and organic extracts against some pathogenic and antagonistic fungi

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In the present research work, *Nicotiana glauca* Graham was used as a potential source of biologically active compounds. The antifungal activity of leaf and flower aqueous and organic extracts (petroleum ether, chloroforme and methanol) was assessed *in vitro* against three phytopathogenic fungi and two antagonistic agents. These target fungi were subjected to the different types of extracts already incorporated into the Potato Dextrose Agar (PDA) medium at various concentrations. Results revealed an important antifungal activity of *N. glauca* leaf and flower aqueous extracts at all concentrations tested (1, 2, 3 and 4%). However, a relative difference in the extent of the response of the same fungal agent to the extracts tested was observed. In fact, Trichoderma *viride* was found to be more sensitive than the other target species, where the radial growth inhibition noted varied from 37.4 to 63.14% depending on aqueous extracts concentrations and the maximum inhibition was obtained with leaf aqueous extracts applied at 1 and 2% concentrations. Moreover, *T. viride* and *Fusarium oxysporum* f. sp. *melonis* were found to be the most sensitive to leaf and flower organic extracts as compared to the other agents. Growth of *T. viride* was inhibited by 33.7% in the presence of chloroforme leaf extract and petroleum ether flower extracts tested at 3000 and 9000 ppm, respectively. Furthermore, the radial growth of *F. oxysporum* f. sp. *melonis* was reduced by more than 31 and 20% with leaf petroleum ether and flower chloroform extracts, respectively, applied at 9000 ppm. *T.* harzianum, *F. oxysporum* f. sp. *tuberosi* and *F. oxysporum* f. sp. *lycopersici* were found to be less sensitive to *N. glauca* organic extracts as compared to aqueous extracts.

Key words: Antifungal activity, aqueous extracts, allelopathy, *Nicotiana glauca* Graham, *Fusarium*, *Trichoderma* and radial growth.

INTRODUCTION

Fungal diseases of cultivated crops remain the principal limitation to increased agriculture production every year. Therefore, protection of plants from pathogens remains a primary concern of agricultural scientists (Guleria and Kumar, 2006). Since the very beginning of their appearance, researchers have succeeded in controlling some devastating diseases by synthetic fungicides. As several synthetic fungicides are highly effective in controlling plant diseases, their negative effect on human and animal health and also on the agro-ecosystem was gradually realized which entailed serious research in developing

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alternative environmentally acceptable (environment-friendly) methods (Sahni et al., 2005). These efforts included biological control, genetic engineering, use of systemic acquired resistance (SAR) with the help of biotic and abiotic agents (Lyon et al., 1995), and biodegradable natural products especially from medicinal plants (Prithiviraj et al., 1996). The use of pesticides of plant origin has been suggested by some workers as alternatives to synthetic chemicals (Amadioha and Obi, 1999; Amadioha, 2000, 2002). Recent studies has confirmed the efficacy of plant extracts in the control of fungal diseases (Amadioha and Uchendu, 2003; Singh et al., 2010), with the view to countering obvious pollution problems in the environment and avoiding the toxic effects of synthetic chemicals on non-target organisms. Organic (Shafique and Shafique, 2008; Bajwa et al., 2008) and aqueous extracts (Bajwa et al., 2001) of many allelopathic plants are known to exhibit antifungal properties. Allelochemicals reduce the germination of spores and the mycelial growth of pathogenic fungi (Bajwa et al., 2003).

The quest for development of new antifungal agents with powerful and wide range of fungicidal activity led us to investigate *Nicotiana glauca* Graham for potentially important antifungal compounds. *N. glauca*, a fast growing shrub or small tree, belongs to the family Solanaceae (Mizrachi et al., 2000). It branches profusely, and can grow vigorously to 3 m, particularly after good rainfall events (Florentine and Westbrooke, 2005). The leaves are stalked, alternate, elliptical to lanceolate or oval, pointed, bluish or greyish-green. The flowers are greenish-yellow, 30 to 40 mm long; many are borne in a lax panicle (Bogdanovic et al., 2006). Studies have demonstrated that *N. glauca* is highly toxic to humans (Mizrachi et al., 2000) and animals (Panter et al., 2000). The first investigation on toxic effects of secondary metabolites extracted from this plant was initiated as early as the 1930's (Steyn, 1934). In the late seventies, the four major pyridine alkaloids, that is, nicotine, anabasine, anatabine and nornicortine were produced from this species (Hawley, 1977; Waller and Edmund, 1978). Several researchers have reported biological activities of *N. glauca* extracts, but a few comprehensive antifungal activities of *N. glauca* have been reported, although it is widely used by traditional healers. Mdee et al. (2009) reported antifungal activity of acetone extracts of *N. glauca* against ten fungal phytopathogens. Soberon et al. (2007) have also reported its antibacterial and cytotoxic effects. Allelopathic activity of *N. glauca* extracts was reported by Heisey and Delwiche (1983), Florentine and Westbrooke (2005) and Alshahrani (2008).

The aim of this investigation is, therefore, is to assess the *in vitro* antifungal activity of aqueous and organic extracts of *N. glauca* leaves and flowers. This allelopathic plant species was tested, as potential source of natural substances biologically active against three phytopathogenic and two antagonistic fungi.

MATERIALS AND METHODS

Fungal agents

Pure culture of two *Trichoderma* species (*Trichoderma harzianum* and *T. viride*) and three *formae speciales* of *Fusarium oxysporum* that is *F. oxysporum* f. sp. *melonis* (FOM), *F. oxysporum* f. sp. *lycopersici* (FOL) and *F. oxysporum* f. sp. *tuberosi* (FOT) infecting melon, tomato and potato, respectively, were obtained from the Laboratory of Phytopathology of the Regional Center of Research in Horticulture and Organic Agriculture, Chott-Mariem (Tunisia). The isolates were obtained from the diseased samples on Potato Dextrose Agar (PDA), purified and maintained at 4°C until use.

Collection of plant material

Fresh and healthy leaves and flowers of *N. glauca* were collected from Tunisian littoral (Monastir, Tunisia). Fresh materials were washed thoroughly with detergent to remove any dust. Washed leaves and flowers were dried in an electric oven at 30°C for 72 h and crushed to make powder.

Extracts preparation

Aqueous extract was prepared by soaking thirty grams of dried leaf and flower powder of the test species in 100 ml of sterilized distilled water for 24 h. Extract was filtered through a double layered muslin cloth followed by Whatman No. 1 filter paper and then passed through 0.22 µm micro-filter pore size to remove bacteria. Filtrates were preserved at 4°C. To avoid any prospective chemical alterations, the extracts were generally used within a week.

Sequential extraction was carried out in organic solvents with rising polarity: petroleum ether, chloroform and methanol. Eighty grams of powder were immersed in the organic solvent for 7 days at room temperature. Organic extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 45 to 50°C, respectively, to remove the petroleum ether, chloroforme and methanol. Samples of 15, 30, and 45 mg were individually dissolved in 2 ml of methanol and then diluted by adding 3 ml of sterilized distilled water, to make final volume of 5 ml, to give three extract concentration (3000, 6000 and 9000 ppm). The stock extract was stored at 4°C and used within four days.

Antifungal bioassays

The antifungal activity against the test pathogens was determined according to the poisoned food technique of Grover and Moore (1962). In fact, PDA medium was prepared and autoclaved at 150°C for 30 min. Appropriate quantities of aqueous extracts (1.5, 3, 5, and 6.25 ml) and distilled water were added to this medium (40 ml), cooled to 45 to 50°C, to get 1, 2, 3 and 4% (w/v) concentrations of leaf and flower aqueous extracts. The control medium received the same quantity (1.5, 3, 5, and 6.25 ml) of sterile distilled water. Stock solution of organic extracts (5 ml) prepared above at 3000, 6000 and 9000 ppm was added to PDA medium. Control received the same quantity (5 ml) of diluted methanol used as control for all bioassays with organic extracts.

The plant extracts were thoroughly mixed with the medium. Ten ml of each medium was poured in each 9 cm diameter sterilized Petri plate. After solidification, mycelial discs of 5 mm diameter were taken with a pre-sterilized cork borer from 5 to 7 days old culture of each tested fungus and were placed in each Petri plate. Each treatment was replicated thrice. Plates were incubated in an incubator at 25 ± 2°C for 3 to 7 days. Fungal growth was measured by averaging the two diameters taken from each colony.

Table 1. Percentage of inhibition of the mycelial growth of fungal agents induced by *Nicotiana glauca* leaf and flower aqueous extracts tested at different concentrations.

	Leaf aqueous extract				Flower aqueous extract			
Fungal agents	1%	2%	3%	4%	1%	2%	3%	4%
TH	23.37^{a}	30.99^{a}	46.63^{b}	33.74^{ab}	11.57^{a}	23.62^{a}	21.76^{a}	19.78^{a}
TV	58.84^{b}	63.14^{b}	50.65^{ab}	44.87^{a}	37.40^{a}	51.17^{b}	56.38^{b}	46.60^{ab}
FOM	22.13^{a}	14.23^{a}	14.74^{a}	18.70^{a}	1.36^{a}	25.66^{b}	24.72^{b}	18.20^{b}
FOL	25.63^{b}	16.45^{a}	19.65^{a}	19.22^{a}	8.31^{a}	14.41^{ab}	12.76^{ab}	19.89^{b}
FOT	22.78^{b}	6.21^{a}	18.66^{b}	5.38^{a}	2.88^{a}	15.63^{b}	19.08^{b}	24.40^{b}

For each fungus tested and each extract type, values (indicating concentrations) affected by the same letters are significantly similar according to Duncan's test at the 0.05 level. Incubation temperature: 25 ± 2ºC; Incubation period: 3-4 days. TH: *Trichoderma harzianum;* TV: *T. viride;* FOM: *Fusarium oxysporum* f. sp. *melonis*; FOL*: F. oxysporum* f. sp. *lycopersici;* FOT: *F. oxysporum* f. sp. *tuberosi.*.

Percentage growth inhibition of the fungal colonies was calculated by applying the following formula (Khanh et al., 2005):

Growth/inhibition (%) = [(Growth in control – Growth in treatment)/ Growth in control] * 100

Statistical analysis

The SPSS statistical methods [predictive analytics software (PASW) statistics 18] were used to calculate the means, standard errors and standard deviations. Statistical analysis one-way ANOVA was applied to the data to determine differences in the three factors tested (Extracts, concentrations and fungi tested, and their interactions) according to a completely randomized factorial design. To check significant differences between the levels of the main factor, Duncan multiple comparison tests at 5% significance were applied.

RESULTS

Effect of *Nicotiana glauca* aqueous extracts on fungal mycelial growth

Results presented in Table 1 revealed an important antifungal activity exhibited by *N. glauca* leaf and flower aqueous extracts at all concentrations tested. However, the response to extracts seems to be different depending on target agents used. Indeed, *T. viride* was the more affected by the extracts tested than the other fungal agents; the recorded radial growth inhibition varied from 37.4 to 63.14%, depending on extracts and concentrations used. Moreover, *T. harzianum* exhibited more sensitivity to leaf than to *N. glauca* flower extracts; the highest radial growth inhibition of 46.63% was obtained with leaf aqueous extracts applied at 3%. These results revealed the inhibitory effect of aqueous extracts of this allelopathic plant exhibited against these both antagonistic agents.

Also, as shown in Table 1, the mycelial growth of the three *F. oxysporum formae speciales* tested seems to be less affected by *N. glauca* aqueous extracts as compared to *Trichoderma* species. In fact, the maximum allelopathic stress (25.63%) induced by leaf aqueous extract at 1% concentration was recorded in *F. oxysporum* f. sp. *lycopersici*. In contrast, flower aqueous extracts exhibited less inhibitory effects (of about 21.14%) on the mycelial growth of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tuberosi* at the highest concentration tested (4%).

Effect of *Nicotiana glauca* organic extracts on fungal mycelial growth

The effects of *N. glauca* leaf and flower organic extracts on the radial growth of the three phytopathogenic and the two antagonistic fungi tested are presented in Table 2. In fact, the mycelial growth of *T. harzianum* was inhibited on PDA medium amended with the leaf and flower organic *N. glauca* extracts at all concentrations tested. The highest inhibition of about 33.7% was recorded in the presence of chloroforme leaf extract and petroleum ether flower extract applied at 3000 and 9000 ppm, respectively. The response of *T. viride* to leaf organic extracts of N. gluaca was slightly different as compared to *T. harzianum*. The mycelial growth of *T. viride* was slightly inhibited or even stimulated in the presence of flower organic extracts. The addition of the petroleum ether fraction at 9000 ppm induced increased by 6.46% the radial growth of this fungus.

A relatively higher inhibitory activity was exerted by *N. glauca* leaf and flower organic extracts against *F. oxysporum* f. sp. *melonis* in all the concentrations used. The pathogen radial growth was reduced by more than 31 and 20%, with leaf petroleum ether extract and flower chloroform extract, respectively, applied at 9000 ppm. However, *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tuberosi* were found to be less sensitive to *N. glauca* organic extracts as compared to *F. oxysporum* f. sp. *melonis*.

The addition of relatively increasing or decreasing

Table 2 Percentage of inhibition of the mycelial growth of fungal agents induced by *Nicotiana glauca* leaf and flower organic extracts tested at different concentrations.

Fungal agents	Organic extract (ppm)	Leaf organic extract			Flower organic extract		
		3000	6000	9000	3000	6000	9000
	Petroleum ether	8.65^{b}	5.48^{a}	19.01^{c}	14.13^{a}	16.65^{a}	33.98^{b}
TH	Chloroform	33.42^{c}	12.96^{a}	27.39^{b}	24.79^{b}	8.02^{a}	14.11^{ab}
	Methanol	24.46^{b}	8.07^{a}	10.64^{a}	19.88^{b}	16.15^{ab}	11.79^{a}
	Petroleum ether	10.00^{a}	16.79^{a}	17.47^{a}	-5.46^{a}	-1.50^{a}	-6.46^{a}
TV	Chloroform	14.62^{b}	2.38^{a}	4.37^{a}	12.73^{a}	-0.27^{b}	9.42^{a}
	Methanol	0.76^{a}	2.39^{a}	2.91^{a}	-3.37^{a}	7.88^{b}	6.87^{ab}
	Petroleum ether	8.53^{a}	15.88^{b}	31.38^{c}	12.78^{ab}	6.23^{a}	19.00^{b}
FOM	Chloroform	14.33^{b}	10.10^{ab}	5.39^{a}	11.62^{a}	8.92^{a}	20.56^{b}
	Methanol	-0.01^{a}	14.30^{b}	2.28^{ab}	11.62^{b}	1.95^{a}	4.67^{a}
	Petroleum ether	6.83^{a}	-0.84^{a}	2.94^{a}	4.95^{a}	-7.48^{b}	-0.06^{a}
FOL	Chloroform	3.70^{a}	-5.88^{a}	0.47^{a}	-7.15^{a}	7.74^{b}	9.21^{b}
	Methanol	1.40^{a}	-5.89^{a}	-1.50^{a}	-1.90^{a}	-14.96^{a}	0.57^{a}
	Petroleum ether	7.49^{a}	-4.08^{b}	11.49^{a}	1.60^{a}	8.62^{a}	-14.16^{b}
FOT	Chloroform	10.27^{a}	-4.92^{b}	11.36^{a}	6.96^{a}	8.87^{a}	18.20^{a}
	Methanol	-10.92^{a}	14.04^{b}	21.02^{b}	8.01^{a}	-4.93^{a}	7.14^{a}

For each fungus tested and each extract type, values (indicating concentrations) affected by the same letters are significantly similar according to Duncan's test at the 0.05 level. TH: *Trichoderma harzianum;* TV: *T. viride;* FOM: *Fusarium oxysporum* f. sp. *melonis*; FOL*: F. oxysporum* f. sp. *lycopersici;* FOT: *F. oxysporum* f. sp. *Tuberosi*

concentrations of *N. glauca* leaf and flower organic extracts caused depression or stimulation of fungal growth of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tuberosi.* In fact, as clearly demonstrated in the present screening, leaf methanol extracts applied at 9000 ppm exhibited the highest inhibitory (of about 21%) effect against *F. oxysporum* f. sp. *tuberosi*. However, the radial growth of this fungus was enhanced by 10.92% with this same extract when used at the lowest concentration tested (3000 ppm). *F. oxysporum* f. sp. *lycopersici* was found to be the less sensitive to all *N. glauca* organic extracts as compared to the other *F. oxysporum formae speciales* tested; the recorded percentage of inhibition did not exceed 6.83 and 9.21%, respectively with leaf petroleum ether used at 3000 ppm and the flower chloroform extract applied at 9000 ppm. However, with the flower methanol extracts tested at 6000 ppm, the growth of this pathogen was 14.96% higher than the untreated control.

DISCUSSION

Plants are a repository of various biomolecules involved in their different biological activities (Kiran et al., 2010). In fact, various plant extracts have been examined by different investigators for their antifungal activity with the objective of exploring environmentally safe alternatives of plant disease control (Bajwa et al., 2006).

The results of this conceptual study clearly reflect that *N. glauca* has inherent ability to induce allelopathic effects on the *in vitro* growth of the tested fungal species. The relative intensity of this effect, however, varies with the target fungus, as well as the origin, types and concentrations of the extracts used. The differences recorded in the fungitoxic activity of the extracts tested is likely due to the solubility of the active compound(s) in water or the presence of inhibitors to the fungitoxic principle as noted by Qasem and Abu-Blan (1966), Amadioha (2001), and Okigbo and Ogbonnaya (2006).

According to the previously mentioned results, a strong toxicity of *N. glauca* leaf and flower aqueous extracts against all the tested fungi at all concentrations (1, 2, 3 and 4%) was shown. However, the response to extracts seems to be different depending on target agents used. In fact, *T. viride* was more affected by the aqueous extracts tested than the other fungal agents; the recorded radial growth inhibition varied from 37 to 63%, depending on extracts and concentrations used. The inhibition of the mycelial growth of the sensitive agent may be attributed to the presence and detrimental effects of allelochemicals on cell division, cell elongation and nutrient uptake (Blake, 1985). In contrast, mycelial growth of *T. viride* was slightly inhibited or even stimulated in the presence of flower organic extracts as observed with the petroleum ether fraction used at 9000 ppm which enhanced by 6% of the radial growth of this fungus. Similar phenomena were observed by Mughal et al. (1996) who found that some allelochemicals can enhance fungal growth at different concentrations. The differences in the toxicity of different extracts could be attributed to the presence of the active principles that are extracted by different solvents, which may be influenced by several factors such as method of extraction, type of extracting solvent

and time of harvesting plant materials (Nicolls, 1969; Qasem et al., 1996). In fact, as shown in our study, organic extracts were found to be relatively more effective in decreasing the mycelial growth of *T. harzianum* whereas *T. viride* exhibited greater resistance against allelopathic compounds of *N. glauca.* This difference in *Trichoderma* species response may be attributed to their genetic or physiological differences (Shaukat et al., 1983). Previous studies support also these results as reported by Martinez-Lozano et al. (2000) that *Sargassum filipendula* extracts also exhibited variable inhibitory effects against *Aspergillus* species including *Aspergillus niger, A. flavus* and *A. parasiticus.*

The mycelial growth of the three *F. oxysporum formae speciales* tested seems to be less affected, precisely at the concentrations tested, by *N. glauca* extracts as compared to *Trichoderma* species. Differential sensitivity of fungi to various bio-pesticides may be due, among other factors, to the chemical structure of the active ingredient and or metabolic activity of the target fungus (Viyas, 1984). As demonstrated in our study, the maximum allelopathic stress (inhibition by about 23.5%) induced by *N. glauca* leaf aqueous extract was recorded at 1% concentration. In contrast, at this same concentration, flower aqueous extracts exhibited the lowest (4%) inhibitory effects against all the three phytopathogenic species tested. Pandey et al. (2010) observed a similar phenomenon with *Cinnamomum zeylanicum* extracts. These authors concluded that antifungal substances seem to be more prominently present in the bark as compared to leaves. This difference could be attributed to the presence of variable amounts of bioactive secondary metabolites in different parts of the plant. The composition of these secondary metabolites in turn varies from species to species, climatic conditions, and the physiological stage of plant development (Pandey, 2007).

The addition of relatively increasing or decreasing concentrations of *N. glauca* leaf and flower organic extracts caused depression or stimulation of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *tuberosi* growth. These results are also supported by the fact that some allelopathic substances, as previously reported by Puruis et al. (1985), have variable effects, either inhibitory or stimulatory, when applied at different concentrations. Similarly, Fabry et al. (1996) reported that extracts of *Entada abyssinica*, *Terminalia spinosa*, *Harrisonia abyssinica*, *Ximenia caffra*, *Azadirachta indica*, *Zanha africana* and *Spilanthes mauritiana*, at different concentrations had different effects on the radial growth of *Candida* spp. and *Aspergillus* spp. The findings of the current study are consistent with those of Farooq (2002) on the effects of different concentrations of *Achillea millefolium* extracts on the linear growth of *Macrophomina phaseolina* fungus.

The variation in the antifungal activity of the extracts prepared in several solvents may be attributed to differences in chemical nature of those solvents. It is likely that various types of chemicals were dissolved in different solvents resulting in variable biological activity even in the same plant part or organ extracts when distinct solvents were used. Many examples in the literature support these findings. Indeed, Jabeen et al. (2008) observed differences in the inhibitory effects of aqueous and organic extracts of different *Melia azedarach* parts when tested against *Ascochyta rabiei*. Alkhail (2005) studied the effect of aqueous and ethanolic extracts of *Allium sativum*, *Carum carvi*, *A. indica* and *Eugenia caryophyllus* against *F. oxysporum, Botrytis cinerea* and *Rhizoctonia solani* and found that aqueous extracts exhibited more inhibitory activity to fungal growth than ethanolic extract. Similarly, Mokbel and Hashinaga (2005) found variable antimicrobial activity of n-hexane, ethyl acetate, butanol and methanol extracts of *Citrus grandis* against five species of bacteria and three fungal species (*Botrytis cinerea*, *Rhizopus stolonifer* and *Penicillium expansum*). Zafar et al. (2002) reported that chloroform extract of leaves of *M. azedarach* was active against *F.* chlamdosporum while hexane, ethanol and water extracts were not.

In our study, the inhibitory effect recorded with *N. glauca* extracts may be attributed, in part, to the presence of some alkaloids. In fact, Saunders (1979) signaled that the genus *Nicotiana* has been reported as containing alkaloids in the vacuole. Several alkaloids are able to affect biological functions even at very low concentrations and thus, they exhibit antimicrobial activity (Mahajan et al., 1982; McCarthy et al., 1992; Srivastava et al., 1994; Atta-Ur et al., 1997; Singh et al., 1994, 1999, 2000). Antifungal activity of alkaloids was already reported in several other works including different plants (Maurya et al., 2001; 2002; Ahmed et al., 2004; Annapurna et al., 2004; Chung et al., 2004). For example, Olugbade et al. (1992) showed that alkaloids present in the bulb of *Crinus jagus* possessed antifungal activity against *Candida* and *Aspergillus* spp.

It is concluded from our study that aqueous and organic extracts of various parts (leaf and flower) of *N. glauca* may be used as biofungicides against some pathogenic fungi. In fact, among the tested aqueous extracts, *N. glauca* leaf extract was proved to be the most effective against *F. oxysporum formae speciales* at the lowest concentration. However, their use, at a given dose, may negatively affect growth of some antagonistic fungi as shown with *Trichoderma* species. Thus, further researches are needed concerning target pathogens and the adverse effects on the antagonistic microorganisms, and on the concentrations that may effectively inhibit plant pathogens without harming biocontrol agents.

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