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Fungal metabolites affect growth and photosynthetic pigments of *Lupinus termis.*

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Abstract Five fungal strains (*Aspergillus aculeatus*, *Aspergillus subolivaceus*, *Trichoderma viride*, *Fusarium oxysporium* and *Rhizopus oryzae*) were grown on different plant materials (leaves of *Acacia nilotica*; green tea, fruits of green pepper and pomegranate) and fifteen fungal metabolites were obtained. These fungal metabolites were tested by treating *Lupinus termis* seeds to determine seed germination percentage and root length. Most of fungal metabolites showed inhibitory effect on seed germination. Fungal metabolites obtained from growth of *Aspergillus subolivaceus* on *Acacia nilotica* showed the best results e.g: an increase in seed germination ratio and root length of *Lupinus termis*. So this fungal metabolite was fractionated by diethyl ether and ethyl acetate into four fractions which were used for treating the lupine seeds. The pretreatment of lupine seeds with different extracts showed an increase in the determined growth parameters (number of leaves, shoot length, shoot fresh weight, shoot dry weight, root length and root fresh weight) and a decrease in root dry weight. Also, increment of the content of chlorophyll a, chlorophyll b, chl (a+ b), crotenoids and total pigment was detected in the leaves of treated plants.

Introduction

Lupins whole seed contains protein, lipids, fatty acids, ash, fibre, amino acids, carbohydrates, calcium, phosphorus, zinc, iron, copper and manganese (Fatima, 2010). Also the plant contains substantional amounts of antioxidants including vitamin E, vitamin C, thiamin, riboflavin and niacin (Szczapa *et al.*, 2003; Erbas et *al.*, 2005 and Frias et *al.*, 2005).

Lupine seeds are well known to be a rich source of protein for animal and human nutrition in various parts of the world, not only for their nutritional value (high in protein, lipids and dietary fiber), but also for their adaptability to marginal soils and climates. Human consumption of lupine seeds has increased in recent years. Lupine flour is added for its nutritive value (high protein efficiency ratio), and also to provide functional properties in bakery and pastry products, protein concentrates and other industrial products, as well as for the elaboration of lactose-free milk and yoghurt analogues (Lqari *et al.*, 2002 and Jiménez-Martínez *et al.*, 2003).

In addition, Lupine seeds have antioxidant phytochemicals that have many health benefits including prevention of

various diseases associated with oxidative stress such as cancer, cardiovascular disease, neuro-degeneration and diabetes (Wang and Clements, 2008), and containing alkaloids such as spartein and anagryne, lupine has an important place in pharmaceutical industry (Yildiz, 2011). Lupine is also considered to be a good yielding crop especially in poor and stressed environments, for example, as an alternative to other legumes that cannot perform well enough in winter and dry land (Bhardwaj, 2002)

Fungi are remarkable organisms which are famous for their ability to produce a wide range of bioactive molecules called secondary metabolites. However, these molecules act as enhancers of virulence and are not required for growth or development of the producing organism (Calvo *et al.*, 2002; Shwab and Keller, 2008).

The interest in the secondary metabolites is considerable, as many natural products, they are of medical, industrial and/or agricultural importance. Some natural products are deleterious (e.g., mycotoxins), while others are beneficial (e.g., antibiotics) to humankind (Demain and Fang, 2000).

The Aspergilli are producers of a wide variety of secondary metabolites of considerable medical, industrial, agricultural and economic importance. For example, the antibiotic penicillin is produced by *A. nidulans* and the genes involved in the penicillin biosynthetic pathway have been extensively studied (Inglis *et al.*, 2013).

Fungal metabolites are valuable natural resources for agrochemical development which can modify the growth and the metabolism of plants (Kuramata *et al.*, 2007 and Vinale *et al.*, 2014).

The present study was therefore, undertaken to evaluate the effect of metabolites of fungi on growth parameters and photosynthetic pigments in *Lupinus termis* plants.

Material and methods

Microorganisms

Fungal strains were subjected to full identification using the most recent sophisticated facilities: an imaging analysis system using soft-imaging GbH software (analysis Prover.3.0), at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, EGYPT. The stockculture was maintained routinely on PDA slants. The freshly grown slant cultures at 28±2°C were subsequently used for further work or were stored at 4°C. The slants were subcultured routinely at an interval of 4– 5 weeks.

For preparation of dry plant materials, the plant materials, leaves of *Acacia nilotica* or green tea and fruits of green pepper or fruits of pomegranate were dried in an oven at 60°C for 48 hours and grounded into fine powder.

Solid state fermentation medium (SSF)

SSF medium was prepared according to Trevino-Cueto *et al.* (2007) with slight modification: 1.0 g of dry plant material was placed in a 250 ml Erlenmeyer flask moistened with 4.0 ml basal medium (set of flasks were prepared for each fungus). Flasks were autoclaved at 12°C for 20 min, cooled and inoculated with 2.0 ml spores suspension (2.0×10^7 spores) under aseptic condition. All prepared flasks are incubated in static conditions at 30°C for 5 days.

Preparation of fungal metabolites:

a) Water extract:

As recommended by Mukherjee and Banerjee, (2004), the fungal metabolites were obtained by soaking mouldy substrate with distilled water. The mixture was shaken for 1.0 hour and centrifuged for 10 min at 5000 rpm to remove cells and residual substrate. The supernatant solution was found to contain the fungal metabolites.

b) Total diethyl ether extract:

According to Mukherjee and Banerjee, (2004) and Samuel *et al.*, (2011), the mouldy substrate was soaked in diethyl ether solvent and shaken for an hour. Then the mixture was filtered and the residual substrate was washed

by excess of diethyl ether until the running filtrate became clear. The diethyl ether extract of the fungal metabolites was obtained by drying over anhydrous sodium sulfate and evaporation of diethyl ether.

Furthermore, the residual substrate was re-suspended with distilled water and the mixture was shaken for 1 hour and then was filtrated to give the aqueous extract.

c) Separation of total diethyl ether extract:

The diethyl ether extract was separated into four layers by water using separation funnel. The aqueous layer was then re-extracted by ethyl acetate. However, the final extracts of ether and ethyl acetate were further used for treating *Lupinus* seeds (Mohrig *et al.*, 2010).

Survey of the effect of different fungal metabolites on lupine seeds:

A lot of *Lupines termis* seeds were surface sterilized with 0.1% $HgCl_2$. Twenty seeds were suspended in each fungal metabolite (water extract) and subsequently incubated at room temperature (28±2°C) for 10 hours. Seeds were plated at equidistance on moist blotters according to Jalander and Gachande (2012). At the same time, control was also maintained with distilled water for each variety. After seven days of incubation time, germination percentage and root length were measured.

Growth parameters in plant:

A homogenously-sized lot of *Lupinus termis* seeds were selected and surface sterilized by soaking in 0.01 % $HgCl_2$ solution for 3 minutes. After washed thoroughly with distilled water, the seeds were then divided into equal groups, each one contains 100 seeds. Before sowing, each group was soaked for 10 hours in different fungal metabolites extracts (water, ether, ethyl acetate and aqueous extract) each with two levels 100% and 50%. In addition, another group was soaked for the same time in water to serve as a control. All groups of the seeds were cultivated in pots (30 cm in diameter) containing equal amounts of homogenously mixed soil (sand: clay, 1:2 v/v). Ten seeds were sown in each pot and irrigated as usual practice by adding equal amounts of water to each pot. All plants were exposed to normal day length and natural illumination. Super phosphate and urea fertilizers were added to the soil during the first week of cultivation. Samples of stage I (vegetative stage) were taken up for analysis after 30 days growth, while stage II which representing the flowering stage, samples were collected after 60 days growth for analysis.

The collected samples at the two stages were used for assessment of growth parameters (number of leaves/plant, number of nodes/plant, shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight and root dry weight), as well as photothynthetic pigments.

The plant photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined using the spectrophotometric method as recommended by Arnon (1949) for chlorophylls and Horvath *et al.* (1972) for carotenoids as adopted by Kissimon (1999).

Results and discution

Fifteen fungal metabolites were obtained from the growth of five different fungal strains; belong to four genera of *Aspergillus*, *Fusarium*, *Rhizopus* and *Trichoderma*, on different plant material (Table 1). These fungal metabolites were used for treating the *Lupinus termis* seeds. However, most of these fungal metabolites showed inhibitory effect on the seed germination as shown in Table 2. This inhibition of fungal filterates may be due to the presence of toxic metabolites as reported by Haikal, (2008) and Jalander and Gachande, (2012). The production of secondary metabolites by fungi is known to degrade seed quality and reduce the seed viability (Deepavali and Nilima, 2014). Jalander and Gachande (2011a) reported that the inhibition effect of culture filtrates of *Fusarium oxysporum* f.sp. *udum* on different varieties of pigeonpea and the same authors (2011b) also reported that the effect of culture filtrates of rhizosphere fungi of pigeonpea on seed germination and seedling growth of pigeonpea varieties and they found the

inhibition of seed germination and reduction in root-shoot length of seedlings.

Table (1): Growth of different fungal strains on different plants materials.

Parameters / Treatment	*Aspergillus aculeatus*	*Aspergillus subolivaceus*	*Trichoderma viride*	*Fusarium oxysporium*	*Rhizopus oryzae*
Green tea	++++	+++	+++	+++	++++
pomegranate	+++	No growth	No growth	No growth	No growth
Acacia nilotica	+++	++++	+++	++++	++++
Allium sativum	++++	No growth	No growth	+	+++
Green pepper	+++	No growth	No growth	No growth	No growth

(+): low growth, (+++): moderate growth, (++++): high growth.

In this study the maximum seed germination (95%) was observed in seeds treated with fungal metabolites obtained by the growth of *Aspergillus subolivaceus* on *Acacia nilotica* so this filtrate was used in the next experiments. In this respect, the maximum seed germination (93.57%) was observed in *Aijung rice* seeds treated with *Trichoderma viride* metabolites (Islam and Borthakur, 2012). Alwathnani and Perveen, (2012) also reported maximum seed germination (93%) of tomato seeds treated with *Nostoc linkia* filterate.

Table (2): Effect of fungal metabolites on seed germination of 7 day seedlings.

Parameters / Treatment	Seed germination (%)	Root length (cm)
Control	95	9.8
Aspergillus aculeatus+ green tea	70	8.9
Aspergillus aculeatus+ pomegranate	35	9.0
Aspergillus aculeatus+ *Acacia nilotica*	65	7.6
Aspergillus aculeatus+ *Allium sativum*	40	4.3
Aspergillus aculeatus+ Green papper	60	9.2
Aspergillus subolivaceus+ Green tea	70	8.8
Aspergillus subolivaceus+ *Acacia nilotica*	95	9.7
Trichoderma viride+ Green tea	55	6.7
Trichoderma viride+ *Acacia nilotica*	70	9.5
Fusarium oxysporium+ Green tea	70	5.9
Fusarium oxysporium+ *Acacia nilotica*	75	8.2
Rhizopus oryzae+ Green tea	65	9.1
Rhizopus oryzae+ *Acacia nilotica*	60	5.6
Rhizopus oryzae+ *Allium sativum*	75	6.5

Growth parameters:

The obtained results in Table 3 showed that, at vegetative stage, a non-significant increase was detected in shoot parameters, in response to all treatments with exception of plants treated with water extract (50%), ether extract (100%& 50%) which showed significant increase in shoot fresh weight. Regarding the changes in root parameters, a general insignificant increase was also observed for root length and root fresh weight while, an insignificant decrease was recorded for root dry weight. During flowering stage, all determined shoot and root

parameters insignificantly increase as compared with controls except root dry weight which showed non- significant decrease.

The obtained results are in harmony with those results of Nafie, (2003) and Khan *et al.*, (2005). Recently, Alwathnani and Perveen, (2012) reported an increase in the whole plant fresh weight and dry weight for the plants treated with *Nostoc linkia* filterate. Moreover, yeast application caused a significant increase in growth parameters (Khalil and Ismael, 2010). Furthermore, in general treatments with concentration of 50% increase in growth parametrers more than that of concentration 100%, and such results are in agreement with that obtained by Haikal, (2008) who used different concentrations of fungal metabolites for treating the soybean seeds, and his final results showed that metabolites with 50% concentration increased growth more than that 100%.

Table (3): Effect of different fungal extracts on growth parameters of *Lupinus termis* plant.

Stage	Treatment	No. of leaves	No. of nodes	Shoot			Root		
				Length	F. wt	Dry wt	Length	F. wt	Dry wt
Vegetative stage	control	12	11	17.48	8.890	1.235	5.98	0.786	0.173
	water extract (100%)	12	11	20.66	9.536	1.168	7.14	0.818	0.116
	Water extract (50%)	13	12	19.68	10.247*	1.187	9.14	1.17	0.123
	Ether extract (100%)	13	12	19.56	10.127*	1.159	6.48	1.181	0.123
	Ether extract (50%)	13	12	20.92	10.179*	1.243	6.88	1.241	0.13
	Ethyl acetate extract (100%)	12	11	20.54	8.969	1.013	6.7	0.869	0.082
	Ethyl acetate extract (50%)	13	12	18.76	9.862	1.127	6.84	1.292*	0.183
	Aqueous extract (100%)	12	11	18.32	8.727	0.997	7.64	1.258*	0.157
	Aqueous extract (50%)	12	11	19.58	8.847	1.011	6.3	0.920	0.103
	LSD	1.09	1.09	3.492	1.157	0.3	2.063	0.461	0.089
Flowering stage	control	23	22	54.433	18.565	3.416	11.667	7.718	1.128
	water extract (100)	25	24	67.100	24.586	3.996	12.767	7.783	1.018
	Water extract (50%)	25	24	66.63*	26.112	4.699	11.700	8.011	1.039
	Ether extract (100%)	25	24	67.20*	24.326	4.243	15.767	7.714	1.000
	Ether extract (50%)	26	25	59.633	26.148	4.687	13.433	7.874	1.021
	Ethyl acetate extract (100%)	24	23	62.433	22.280	3.748	11.567	7.776	1.066
	Ethyl acetate extract (50%)	26	25	63.800	22.460	3.740	12.367	7.867	1.088

Aqueous extract (100%)	23	22	55.00	24.916	3.940	14.867	7.867	1.068
Aqueous extract (50%)	25	24	65.500	25.006	4.342	11.300	7.903	1.078
LSD	2.776	2.776	11.433	13.573	2.063	4.43	5.89	0.55

(*)= significant increase or decrease at 0.05 LSD

Photosynthetic pigments

The results presented in Table 4 showed that at vegetative stage, treatments with extracts of ether (50%), ethyl acetate (100% & 50%) and aqueous extract (100% & 50%) caused significant increase in all pigments content. All other treatments, except water extract (100%) which led to a non-significant decrease in Chl. (a) and Chl. (b), caused insignificant increase in photosynthetic pigments content.

The data obtained during the flowering stage in Table 4, showed that with exception of water extract (50%) treatment that caused non- significant decrease for carotenoids content and ether extract (100%) which caused non- significant decrease in Chl. (b), (Chl. a+ Chl. b), carotenoid and total pigments content, the other treatments generally caused insignificant increase for all photosynthetic pigments.

Generally, the all treatments increased the content of Chl. (a), Chl. (b), (Chl.a+ Chl.b), carotenoid and total pigments in the treated plants. This result was similar to the result obtained by Hwang *et al.* (2011) who reported an increase in the contents of chlorophyll a and chlorophyll b, total chlorophyll, and carotenoid in leaf blades in plants treated with fungal culture filtrate. Recently, nursery treatments with *A. niger* isolates improved the leaf chlorophyll a, chlorophyll b and total chlorophyll in tomato plants (Anwer and Khan, 2013). This result is also supported by the positive effect of yeast on chl a and chl b as reported by Hayat, (2007) and Stino *et al.* (2009).

Table (4): Effect of different fungal extracts on photosynthetic pigments (mg/g dry weight) of *lupinus termis* plant.

Stage	Treatment	Chl. a	Chl. b	Chl. a+ chl. b	Carotenoids	Total pigments
Vegetative stage	control	3.156	4.481	7.637	2.196	10.525
	water extract (100%)	3.124	4.115	7.239	2.284	10.181
	Water extract (50%)	3.335*	4.419	7.754	2.287	10.774
	Ether extract (100%)	3.532*	4.562	8.094	2.423	11.412
	Ether extract (50%)	3.611*	6.205*	9.816*	4.433*	14.711*
	Ethyl acetate extract (100%)	4.017*	6.417*	10.435*	5.301*	16.195*
	Ethyl acetate extract (50%)	3.919*	7.118*	11.037*	5.255*	16.829*
	Aqueous extract (100%)	3.939*	6.991*	10.93*	5.751*	17.182*
	Aqueous extract (50%)	3.886*	8.437*	12.322*	5.596*	18.831*
	LSD	0.047	1.395	1.332	0.488	3.675
Flowering stage	control	2.346	2.112	4.457	1.567	6.024
	water extract (100)	2.619*	2.170	4.789	1.569	6.358
	Water extract (50%)	2.456	2.162	4.618	1.521	6.105
	Ether extract (100%)	2.425	1.807	4.164	1.524	5.655
	Ether extract (50%)	2.478	2.593	5.070	1.682	6.751
	Ethyl acetate extract (100%)	2.363	2.255	4.378	2.288*	6.667
	Ethyl acetate extract (50%)	2.571*	2.549	5.120	3.594*	8.714
	Aqueous extract (100%)	2.634*	2.297	4.931	2.974*	7.904*
	Aqueous extract (50%)	2.356	2.970	5.325	2.400*	7.725*
	LSD	0.145	0.645	0.875	0.543	1.27

(*)= significant increase or decrease at 0.05 LSD.

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الأيض الفطري يؤثر على نمو وأصباغ نبات الترمس

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تم تنمية خمس سلالات من الفطريات على المواد النباتيه المختلفه (اوراق السنط الشاي الاخضر ثمار الفلفل الاخضر و لب الرمان) وتم الحصول على خمسة عشر أيضا فطريا.

تم اختبار الأيض الفطري على نمو بذور الترمس و قد اوضحت النتائج ان معظم الأيض الفطرى أدي الي تقليل نمو البذور لكن الأيض الفطري الناتج عن نمو فطرة *Aspergillus subolivaceus* على اوراق نبات السنط *Acacia nilotica* أدي الي افضل النتائج من زيادة نسبة نمو البذور وزيادة طول الجذر لذلك تم فصل مكونات هذا الأيض باستخدام diethyl ether و ethyl acetate الى اربعة اجزاء (مستخلصات) تم استخدامها لمعاملة بذور الترمس.

ادى نقع بذور الترمس بالمستخلصات الفطريه لمدة عشر ساعات الى زيادة عدد الاوراق و طول الساق ووزن الساق الطازج ووزن الساق الجاف و طول الجذر ووزن الجذر الطازج بينما ادت هذه المعاملات الى تقليل وزن الجذر الجاف. كما ادت هذه المعاملات الى زيادة محتوى كلوروفيل أ وكلوروفيل ب وكلوروفيل (أ+ ب) والكاروتين وكذلك المحتوى الكلي الكلوروفيلي فى النباتات الناتجة وذلك خلال مرحلة النمو (الخضرية- الزهرية).