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Effect of the mycelium, diffused substances and extracts of the fungus *Cladosporium herbarum* (Pers.) Link on the *in vitro* and *in vivo* growth of grapevine (*Vitis vinifera* L.) cv. Cabernet Sauvignon

by

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S u m m a r y : The fungus *Cladosporium herbarum* (Pers.) Link was cocultured with plantlets of grapevine (*Vitis vinifera* L.) cv. Cabernet Sauvignon *in vitro* and *ex vitro*. The mycelium of the fungus stimulates growth of the plantlets. This is probably exerted by the diffusion of some growth and/or nutritional substance(s) of unknown chemical nature. In another trial filtrates of the fungus and fungus extracts were added to the culture medium of grapevine plantlets in different concentrations. The fungus extracts stimulated growth of plantlets at low concentrations and inhibited growth at high concentrations.

K e y w o r d s : Vitis vinifera, Cladosporium herbarum, in vitro culture, shoot/root ratio.

Introduction

It is well known that some microorganisms synthesize substances which have the chemical nature of growth regulators controlling growth and development of higher plants (Arshad and Frankenberger 1991). Auxin production by a wide group of soil microbial isolates has been demonstrated by many workers (Arshad and Frankenberger 1993). Indole-3-acetic acid is a common product of the L-tryptophan metabolism of soil fungi (Gruen 1959; Frankenberger and Brunner 1983). Some cytokinins have been found in the supernatant of bacteria cultures (Nieto and Frankenberger 1988; 1989 a) and in soil filtrates previously additioned with purine ring constituents and isoprenoid compounds (Nieto and Frankenberger 1989 b). Gibberellins have been discovered from the "bakanae" rice disease produced by *Gibberella fujikuroi* (Kurosawa 1926), and methods for great gibberellin production have been developed (Borrow *et al.* 1955). Gibberellin-like substances have also been isolated from some bacteria (Lee *et al.* 1970). At present it is known that several microorganisms have the capacity to synthesize gibberellins in axenic culture in different ranges of concentration (Rademacher 1994). Other fungi, bacteria and algae have been cited as ethylene (Lynch 1972; Da Silva *et al.* 1974; Lynch and Harper 1974 a, b; Primrose 1976, 1979; Smith 1976; Thomas *et al.* 1977 Arshad and Frankenberger 1988, 1989, 1990) and jasmonate producers (Aldridge *et al.* 1971; Miersch *et al.* 1991). Recently, cyclo (L-tryptophyl-L-phenylalanyl) has been isolated from cultures of *Penicillium* spp. as growth regulator of higher plants (Kimura *et al.* 1996).

In *Vitis vinifera* L., the existence of endotrophic mycorrhiza has been described in relation to the grapevine nutrient status (Possingham and Groot Obbink 1971); *in vitro*-cultured Kober 5 BB plants were used to study the effect of inoculation with vesicular-arbuscular mycorrhizal fungi (Schubert *et al.* 1987).

Roots of *V. riparia* infected with the fungus *Aureobasidium pullulans* showed an internodal stimulation of growth, shoot branching and development of adventitious roots. Some of these effects have been produced without a direct contact between fungus and plant and even with cell-free extracts added to the *in vitro* culture medium (Blaich and Ruster 1979).

Materials and methods

D e s c r i p t i o n a n d c u l t u r e o f t h e f u n g u s : The fungus belongs to the class Deuteromycetes, the sexual expression is named *Mycosphaerella tassiana* (de Not) Johanson, and the conidic, asexual expression is named *Cladosporium herbarum* (Pers.) Link.

The fungus has a limited parasitic capacity, normally confined to the stomatal chambers where it produces the death of only a few cells (O'Donnell and Dickinson 1980). In the field, the fungus is saprophytic with a high degree of sporulation (Hewitt 1988).

After isolation, samples of the fungus have been cultured in Petri dishes containing glucose potato agar (pH=6.5) supplemented with some drops of lactic acid in order to inhibit bacteria growth. After purification through some cultures, the fungus was incubated at 25 °C for one week and then placed at room temperature. When the colonies reached 4 cm in diameter, 5 mm agar discs were excised and incorporated in a medium where grapevine plantlets were cultured.

The colonies presented a dark green colour with a velvety appearance produced by a great number of fructifications.

I n v i t r o a n d *e x v i t r o* c u l t u r e o f i n f e c t e d p l a n t l e t s . E f f e c t o f t h e m y c e l i u m : Green one-node cuttings were cultured in the medium of Galzy (1964) under long day condition (16 h; 45 µmol quanta·m^{-2}·s^{-1}) at 25±2 °C.

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Table 1

Effects of the fungus *C. herbarum* on leaf number, shoot length, number of primary roots, length of the longest root, leaf area, specific leaf area, dry weight (DW) of shoots, leaves, petioles and roots and the leaf/root ratio on the growth of Cabernet Sauvignon grapevines

	in vitro		*ex vitro*	
	without fungus	with fungus	without fungus	with fungus
Shoot length (cm)	5.1 a	4.6 a	8.1 a	7.5 a
Leaf number	9.1 a	8.0 b	4.8 b	5.5 a
Leaf area (cm^2)	16.2 a	15.3 a	40.5 a	25.6 b
Specific leaf area (mg DW·cm^{-2})	1.3 b	3.1 a	2.6 b	3.0 a
Number of primary roots	2.0 b	2.8 a	5.4 a	3.5 b
Length of the longest root (cm)	12.4 b	15.7 a	13.4 b	18.9 a
DW of roots (mg)	11.8 b	57.1 a	162.1 a	187.6 a
DW of shoots (mg)	7.4 b	19.6 a	67.7 a	56.9 b
DW of leaves (mg)	21.4 b	42.9 a	105.4 a	74.6 b
DW of petioles (mg)	1.7 b	3.2 a	8.0 a	5.5 a
Leaf/root ratio (mg·mg^{-1})	2.49 a	1.11 b	1.10 a	0.71 b

Means followed by the same letter are not significantly different at the 0.05 level.

The inoculated agar discs were aseptically placed at the center of 350 ml flasks containing four 3-4 nodal plantlets.

In a first trial two treatments were carried out: a) 13 flasks with 4 inoculated plantlets, and b) 13 flasks with 4 non-inoculated plantlets.

The plantlets were inoculated 32 d after the one-node cuttings culture, when they had 3-4 nodes.

30 d after the inoculation the first evaluation was made with 7 flasks of each treatment. The parameters evaluated were: leaf number, shoot length, number of primary roots; length of the longest root, dry weights (DW) of shoots, leaves, petioles and roots; leaf area; specific leaf area and the shoot/root ratio (Tab. 1).

The plantlets of the 12 remaining flasks (6 with and 6 without fungus) were removed from the flasks, their roots rinsed with water and treated for 5 s with a fungicide containing chlorotalonyl 50 % (isophthalonitrile tetra-chlore, 15 mg·l^{-1}). After this treatment the plantlets were placed in plastic pots (6 cm in diameter and 7 cm depth) containing perlite which had been previously sterilised.

Plantlets acclimation took place under greenhouse conditions and then in the field under antiaphid covers. The acclimation technique is described by RIQUELME *et al.* (1991).

During the first week the plantlets were covered with transparent plastic film; then they were gradually exposed to environmental conditions for 2 weeks. Temperature ranged from 24 to 27 °C during the day and from 19 to 23 °C during the night. In the last week of acclimation plantlets were in the field under antiaphid tissue. The plantlets were periodically watered with MURASHIGE-SKOOG (1962) mineral solution. After acclimation the plantlets were harvested and analysed using the parameters described above (Tab. 1).

Another trial was performed to find out if the action of the fungus could be induced by diffusion of some growth factors in the culture medium or by direct contact of the fungus with the roots of the plantlets (mycorrhizal effect). A glass cylinder, 7 cm in length and 0.7 cm in diameter, with three 0.5 cm segments was placed in the center of each flask to allow a continuous contact of the culture medium with that of the cylinders.

The glucose-potato-agar discs carrying the fungus were placed on the surface of the medium in the cylinders. By this the expansion of the fungus toward the roots of the plantlets was impeded. The inoculation was carried out 35 d after the culture of the one-node cuttings, when the young plantlets (3-4 nodes) developed roots.

Four treatments with 8 flasks containing 4 plantlets each were made: 1) without fungus and cylinders; 2) without fungus, with cylinders; 3) with fungus, without cylinders, and 4) with fungus and cylinders (Tab. 2). The material was exposed to 16 h light at 24 °C for 30 d from the culture of the fungus. After treatments the parameters of Tab. 2 were evaluated.

Effect of diffused substances: An agar disc (5 mm in diameter) which had been previously inoculated with the fungus was put in each of nine 250 ml flasks containing 100 ml of liquid culture medium according to GALZY (1964). The flasks were placed in a thermostated rotating bath (25 °C) for 6 d. The supernatant of 3 flasks was carefully sterilised through a membrane (0.2 µm pore size).

Grapevine plantlets were previously cultured in a 0.8 % agar medium (GALZY 1964). Three plantlets were cultured per flask containing 45 ml medium. After 44 d of culture the plantlets (having 3-4 leaves and well developed roots) were inoculated with 4 ml of the eluate. In the control flasks 4 ml of the medium of GALZY were added (Tab. 3). The weight of the fungus harvested from 3 flasks was 14.21 g (dry weight: 1.71 g, 12 %).

Preparation of fungus extracts: Fragments of the fungus were cultured in Petri dishes con-

Table 2

Effects of the fungus *C. herbarum*, in contact or not with the roots of plantlets of Cabernet Sauvignon, on shoot length, leaf number, leaf area, specific leaf area, DW of roots, shoots, leaves and petioles and leaf/root ratio

	WOF/WOC	WOF/WC	WF/WOC	WF/WC
Shoot length (cm)	7.8 a	7.7 a	8.0 a	8.0 a
Leaf number	10.0 a	9.2 a	10.4 a	10.7 a
Leaf area (cm^2)	30.0 ab	25.0 b	29.5 ab	33.3 a
Specific leaf area (mg DW·cm^{-2})	1.75 a	1.83 a	2.05 a	1.73 a
DW of roots (mg)	24.4 b	22.3 b	43.0 a	42.3 a
DW of shoots (mg)	19.9 b	20.0 b	28.6 a	28.2 a
DW of leaves (mg)	51.4 ab	45.2 b	59.8 a	56.4 a
DW of petioles (mg)	3.5 bc	3.2 c	4.6 a	4.2 ab
Leaf/root ratio (mg·mg^{-1})	2.55 a	2.85 a	2.21 a	2.11 a

WOF: without fungus; WF: with fungus; WOC: without cylinders; WC: with cylinders.
Means followed by the same letter are not significantly different at the 0.05 level.

taining glucose-potato-agar for 15 d at 25 °C. When the colonies had developed up to 4-5 cm in diameter, one disc (5 mm in diameter) of inoculated agar was placed in each flask containing 100 ml of sterilised culture medium of GALZY. For one month the material was exposed to room temperature, diffuse light and regular shaking.

Two types of extracts were prepared: a) One extract representing the liquid of filtration, and b) another extract made with the mass of the fungus.

a) The liquid of filtration (167 ml) was purified with the technique (FRANKENBERGER and BRUNNER 1983) used for purification of indoles before evaluation by HPLC. The pH of the liquid was adjusted to 8. Then, it was partitioned three times with ethylacetate which was discarded. The aqueous fraction was acidified to pH 3 and then partitioned again with ethylacetate. The aqueous phase was discarded.

The extract of ethylacetate was divided in two fractions of 100 and 67 ml. Each fraction was evaporated to dryness. The residue of 100 ml was rehydrated with 50 ml distilled water and that of 67 ml was diluted again with 67 ml of distilled water. The first extract was named "double extract" and the other "simple extract".

b) The mass of the fungus (1.6 g dry weight) was immersed in liquid nitrogen; then it was crushed and treated with methanol at low temperature for 24 h. After filtration, the fungus was treated again with methanol for 1 h at low temperature. Both extracts were combined. After evaporation to dryness *in vacuo*, the residue was dissolved with a buffer (phosphate, pH 8) and then partitioned with ethylacetate.

The technique of purification described above was used just to obtain simple and double extracts. Five treatments were performed: 1) Control, addition of distilled water; 2) simple filtrate (SF) with solution of simple filtrate; 3) double filtrate (DF) with solution of filtrate of double concentration; 4) simple macerate (SM) with solution of the simple extract of the fungus and 5) double macerate (DM) with solution of the double concentration extract of the fungus. In all cases 4 ml of solution were added to each flask.

Each treatment consisted of 12 flasks containing 3 plantlets with 3-4 nodes with 45 ml of the Galzy medium.

The results were statistically analysed by the "Analysis of Variance". The test of Tukey was employed to analyse differences between media.

Results and Discussion

One week after inoculation shoots of treated plants had a red colour probably due to anthocyanine synthesis. From the 10th day onward a stimulation of root growth and a higher thickness of growing shoots was observed.

At the end of the *in vitro* culture of the first assay, the roots of plantlets were covered with the mycelium of the fungus but only in the region adjacent to the "neck" of plantlets. Data of Tab. 1 show that the fungus produced a significant reduction of leaf number, but a stimulation of growth and development of primary roots.

During the *ex vitro* phase (acclimation, Tab. 1) a significant increase of leaf number with a decrease of internode length and a significant reduction of the number of primary roots and a higher degree of lignification was observed. Nevertheless, the DW of shoots and leaves were significantly smaller in the inoculated plantlets without modification of the DW of roots.

Leaf area was increased in the control plantlets but with a higher specific leaf area in the inoculated plantlets. The leaf/root weight ratio was similar to that observed *in vitro*, but with a significant difference in relation to the control.

In the second assay (Tab. 2) we did not observe any contact between the mycelium of the fungus and the roots of the plantlets, due to the presence of the glass cylinders. Thus we observed only a superficial development (1-2 mm in the culture medium inside the cylinders) of the mycelium.

Data of Tab. 2 clearly show that the action of the fungus takes place by diffusion of some substance(s) in the

Table 3

Effect of the filtrate of the fungus *C. herbarum* on growth pattern of grapevine plantlets, cv. Cabernet Sauvignon

	shoot length (cm)	leaf number	DW of leaves (mg)	DW of shoot (mg)	DW of root (mg)
With filtrate	3.8	15.4	10.9	35.5	19.2
Without filtrate	3.7	16.6	10.8	39.5	24.3

Table 4

Effects of simple and double filtrates and macerates of the fungus *C. herbarum* on growth pattern of *in vitro*-cultured grapevine plantlets, cv. Cabernet Sauvignon

	shoot length (cm)	leaf number	DW of leaves (mg)	DW of shoot (mg)	DW of root (mg)	leaf/root ratio
Control	6.6 bc	9.1 a	37.4 ab	13.1 c	51.2 b	0.99
Simple filtrate	6.8 bc	9.6 a	41.3 a	15.3 bc	47.7 b	1.19
Double filtrate	7.3 ab	10.0 a	44.7 a	16.7 bc	52.2 b	1.17
Simple macerate	8.3 a	8.7 a	49.8 a	27.2 a	144.3 a	0.53
Double macerate	5.8 c	5.5 b	28.2 b	20.7 ab	124.2 a	0.39

Means within columns followed by the same letter are not significantly different at the 0.05 level.

culture medium. This idea is confirmed by the absence of differences between treatments with and without glass cylinders. In both cases the leaf/root weight ratio was the same but was much smaller compared to that of the control. This seems to suggest a greater adaptability to possible water stress in the field once the plantlets have been definitively transplanted.

From the analysis of data (Tabs. 1 and 2) it is evident that the action of the fungus is exerted by the diffusion of some growth and/or nutritional substance(s) of unknown chemical nature.

It is interesting to note that the fungus also exerts a stimulating action in potato. Inoculated plantlets with 2-3 nodes showed higher vigour and a better degree of adaptation to water stress in the field (TIZIO, personal communication).

Data of Tab. 3 show that the filtrate of the fungus did not modify any of the parameters analysed, perhaps due to the low concentration of the filtrate. Nevertheless, there is a tendency to inrease the DW of shoots and roots.

The analysis of data (Tab. 4) shows that the "simple macerate" significantly stimulated shoot growth, DW of shoots and, in particular, that of the roots. "Double macerate" partially inhibited shoot growth, leaf number and DW of leaves, but not that of roots which was significantly higher than that of the control plantlets. The ratio leaf DW/root DW was lowest in the treatments with macerates in relation to that of the control and those of simple and double filtrates.

The data of Tab. 4 show a gradual increase of DW from the control, simple and double filtrates to the highest value obtained with "simple macerate".

The data of this report give no indication on the chemical nature of the growth substance(s) synthesized by the fungus. Nevertheless, the technique of isolation and purification of auxins employed, suggests that the growth substances produced by the fungus could be auxin-like in nature. From a practical point of view it is evident that the "simple macerate" stimulates plantlet growth with a low ratio leaf DW/root DW indicating a better adaptation of plantlets to field conditions.

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