

**Watering and mineral supply effects on plant production, on hydrosoluble metabolites content and on the antifungal properties of three aromatic plants used as spices**

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**ABSTRACT**

*Calamintha baetica*, *Origanum vulgare* and *Cuminum cyminum* have been studied for their capacities as a source of antifungal compounds. One set of these plants grown under water stress. The other set of plants was submitted to different mineral nutrition. The plant material obtained after grinding, were extracted with MeOH or EtOAc, fractionated with organic solvents, and tested for their antifungal properties. *Cladosporium cucumerinum* and several *Fusarium* strains were used as biological targets. NMR was used for studying the metabolites content of *C. baetica* produced under either watering or mineral stress conditions.

**I. INTRODUCTION**

Plants are characterised by the diversity of metabolites produced. Several substances of plant origin have been identified and studied for their biological activities (Corley & Durley, 1994), such as antiviral (Colombo & Bosisio, 1996), bactericidal (Schmeller *et al.*, 1997), antifungal (Gupta *et al.*, 1996), herbicidal and insecticidal (Rageau & Delaveau, 1979, Schmeller *et al.*, 1997).

The aromatic plants *Cuminum cyminum*, *Origanum vulgare* and *Calamintha baetica* traditionally used as spices have preservative properties which could be related with their content of antimicrobial substances. In order to study the capacity to produce antifungal substances, aromatic plants were grown under mineral and water stress conditions, and their extracts were tested against *Cladosporium cucumerinum* and several *Fusarium* strains. We also considered the detection in plant extracts of light stimulated biocidal activity (Heitz & Downum, 1987).

**II. EXPERIMENTAL**

**I. Plant material**

Plants were grown under four watering regimes: 25 (I), 50 (II), 75 (III) and 100% (IV) of field capacity, and four mineral nutrition modalities: application of a water supply solution without K (I), without Ca (II), without P (III), complete nutrition (IV) and free of nutrition (V).

Plants obtained from each modality of stress were extracted with a range of organic solvents of increasing polarity. The extracts obtained, complete and fractionated by column and TLC chromatography, were tested for antifungal activity.

## 2. Bioassays

### 2.1. Fungus growth

For the three species studied the antifungal tests were performed against *Cladosporium cucumerinum*. *Calamintha baetica* was also tested on *Fusarium* strains (*F. culmorum*, *F. solani*, *F. oxysporum f. sp. melonis* 26 race 1, *F. oxysporum f. sp. melonis* Y race 1.2, *F. oxysporum f. sp. melonis* MC2 race 0). *F. oxysporum f. sp. melonis* strains will be referred as *F.o.m.*

Petri dishes were prepared with 20 ml of potato dextrose agar (PDA) and an inoculum of 5 mm of diameter. After incubation for 5 days (*Fusarium*) or 10 days (*C. cucumerinum*) at 23°C, fungus suspensions were prepared by disintegrating the mycelium in an adequate liquid media. Each fungus suspension was filtered through a nylon net (pore of 100 µm) on a buckner/kitasato, under vacuum.

### 2.2. Antifungal tests

The fungus suspension was sprayed on the TLC plates containing both complete and fractionated extracts. After incubation for 72 hours, at 25°C, in a wet chamber, the antifungal activity of the extracts components was detected by the presence of white spots corresponding to the areas of inhibition of fungus growth. For the detection of photoactive substances two similar TLC plates were performed. One was incubated in dark and the other was irradiated for 24 hours. Photoinhibition was detected by comparing the spots formed for dark and for light conditions.

## 3. Water soluble metabolites

For the evaluation of the effects of watering and mineral nutrition on the production of water soluble metabolites, leaves of *Calamintha baetica* were analysed. Leaves (2g fresh mass) were collected at flowering stage, extracted with ethanol: water (80:20 v/v), lyophilised and dissolved in water (3 ml) containing D<sub>2</sub>O (250 µl) as an internal standard. After adjusting to pH 8.0, the suspension was centrifuged for 5 min (10 000 rpm) and the supernatant used to <sup>13</sup>C-NMR spectroscopic measurements, performed on a Bruker AMX-300. Metabolites content are relative to control modalities (IV) of water and mineral stress experiments.

## III – RESULTS

### Extraction Yields

The dried plant material of *Calamintha baetica* was extracted with MeOH or EtOAc before and after grinding. The highest extraction yields were obtained for grinding (results not shown) material extracted with MeOH Fig. 1C. The solvent extraction values of *Origanum vulgare* and *Cuminum cyminum* reached with MeOH or EtOAc were referred in Fig. 1A and 1B). For these two plants MeOH was also the solvent with the best yield extraction.

### Antifungal activity

For the evaluation of antifungal activity either MeOH or EtOAc extracts were tested on several *Fusarium* strains and on *Cladosporium cucumerinum*. The highest inhibition were attained with EtOAc extracts, assayed in the dark. However, MeOH extracts under light conditions showed antifungal activity. The antifungal activity of *C. baetica* extracts against *Cladosporium cucumerinum* was shown in Table 1. Plants subjected to water stress or well watered produced more antifungal substances. The influence of mineral stress on bioactivity changed with plant species (compare Table 1 and Table 2).

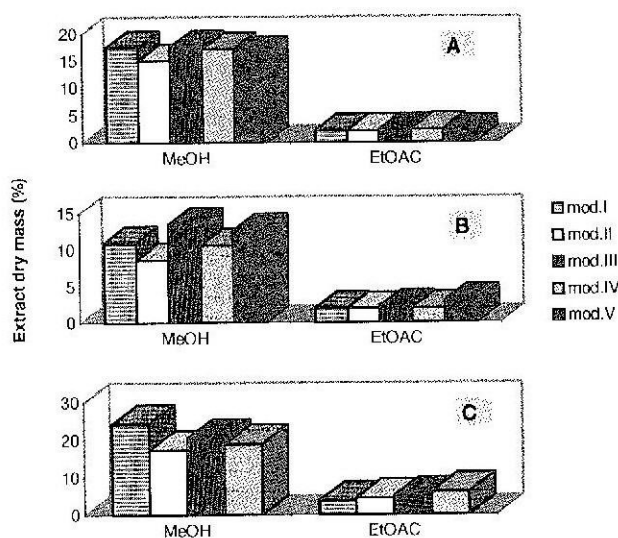


Fig. 1 - Extraction yield, with MeOH or EtOAc, of *Origanum vulgare* (A), *Cuminum cyminum* (B) and *Calamintha baetica* (C), obtained from the mineral stress experiments.

Table 1- Inhibitory effects on *Cladosporium cucumerinum* growth of the *Calamintha baetica* extracts obtained by maceration with MeOH or EtOAc. Plants produced under: A - different watering modalities (I -25%, II -50%, III -75 % and IV -100% of field capacity) or B - mineral nutrition (I-without K, II-without Ca, III-without P, IV-complete nutrition). Percentage of fungi growth inhibition when grown either on dark or after light irradiation: +++++100-80%; ++++80-60%; +++ 60-40%; ++ 40-20%; + 20-5%; (+) 5-1%; -no inhibition.

Extracting Solvents	A - Watering modalities							
	I		II		III		IV	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark
EtOAc	+++++	++++	+++	+++	++++	+++	+++++	++++
MeOH	+++	+	+++	+	++++	-	+++	-

Plant	Extracting solvents	Mineral nutrition									
		I		II		III		IV		V	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
A	E t O A c	++	++	+++	+++	+++	++	+++	++	+++	++
	MeOH	+++	+	+++	+	++	+	++	+++	+++	++
B	EtOAc	+++	++++	+++	+++++	+++	+++	+++	+++++	++	+++
	MeOH	++	+	++	+	+++	++	+++	++	++++	++

For *C. baetica* the best results were obtained when plants were grown without added K or Ca. Otherwise, *Origanum vulgare* and *Cuminum cyminum* showed maximum bioactivity when plants were grown with complete nutrients supply or Ca deficiency.

*C. baetica* extracts from the mineral and water stress experiments produced a slight inhibitory effect on *Fusarium*'s growth. *Fusarium* strains showed different sensitivity to the extracts, being *F. culmorum* (F1) and *F.o.m.* MD2 race 0 (F5) the less affected, *F. solani* (F2) and *F.o.m.* Y, race 1.2 (F4) were slightly affected. *F.o.m.* 26, race 1 (F3) was the most affected fungus irrespective to the treatments (Fig. 2).

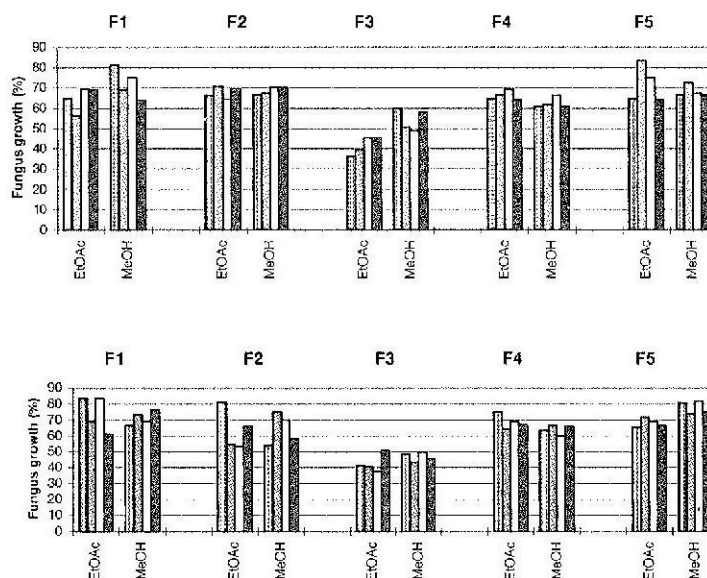


Fig. 2 - Effect of *Calamintha baetica* extracts obtained by maceration with methanol (MeOH) or ethyl acetate (EtOAc), on the growth of the fungi *Fusarium culmorum* (F1), *F. solani* (F2), *F.o.m.* 26, race 1 (F3), *F.o.m.* Y, race 1.2 (F4) and *F.o.m.* MC2 race 0 (F5). Plants submitted to: A - Irrigation with 25% (■), 50% (▨), 75% (□) and 100% (▩) of field capacity; B - Mineral nutrition without K (■), without Ca (▨), without P (□) and all nutrients (▩).

## Hydrosoluble metabolites

Accentuated water stress (modalities I and II) led to a slight increase of soluble sugars (sucrose, glucose and fructose) and a decrease on organic acids and aminoacids. Nitrogen compounds of low molecular mass that, in other species act as an osmotic protectant, were not detected. The soluble sugars found in the plants grown under the mineral stress modalities I and II, were similar to the control. Plants grown without P have higher sugar concentration. Water stressed plants produced less organic acids and aminoacids (Table 3).

Table 3 - Hydrosoluble metabolites content in leaves of *Calamintha baetica* grown either under water stress (A) (I-25%, II-50%, III-75% and IV-100% of field capacity) or mineral stress (B) (I-without K, II- without Ca, III- without P, IV-complete supply of nutrients). Relative metabolites content having as control (100%) modalities IV of water and of mineral stress experiments. (n.d. - not detected)

Hydrosoluble Metabolites	A - Watering modalities			
	I	II	III	IV
Sugars	107,2	124,9	97,1	100
Organic Acids	50,8	76,9	115,2	100
Aminoacids	60,0	75,0	124,5	100

Hydrosoluble Metabolites	B - Mineral nutrition			
	I	II	III	IV
Sugars	104,7	101,9	135,3	100
Organic Acids	123,0	121,2	122,4	100
Aminoacids	n.d.	n.d.	n.d.	n.d.

## DISCUSSION AND CONCLUSIONS

Bioactive aromatic compounds are produced by plant metabolism as a response to stress. Geographic variation studies with *Origanum vulgare* allowed the establishment of a direct correlation of essential oils content to the soil and climate (Vokou *et al.*, 1993). These information is in accordance to the results obtained in our assays performed with *O. vulgare*, *C. cynimum* and *C. baetica*.

The water deficit conducted to an increase of the soluble sugars and to a decrease of both organic acids and aminoacids. Plants grown without P showed an increase of sugars relative to the other nutrition modalities where no differences were observed. Organic acid contents increases in a similar way in both mineral and water stress.

MeOH provide higher yields of plant extract than EtOAc. The three plant ethyl acetate extracts showed highest efficiency for fungus inhibition when cultured in dark. Although, for the

tests performed for light antifungal activity, MeOH extracts gave the best results. These evidences that different substances were involved in antifungal activity when dark or light were used.

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