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INTEGRATED CONTROL OF WATER HYACINTH BY USING *Alternaria eichhorniae* ISOLATE 5 (AE5) WITH A PHENYLPROPANOID PATHWAY INHIBITOR

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ABSTRACT

The fungus *Alternaria eichhorniae* isolate 5 (Ae5) is being developed as an effective mycoherbicide against water hyacinth in Egypt. In order to improve its virulance, an integration with MDCA, a phenylpropanoid pathway inhibitor, which weakens the plant defence system, was explored. The disease severity induced by Ae5 increased when its formulation was applied to water hyacinth plants pretreated with MDCA. Infection with Ae5 amplified the total phenol concentration in diseased water hyacinth leaves whereas MDCA reduced it. Plants treated with both Ae5 and MDCA contained level of total phenols comparable to that in the untreated control plants. Anatomically, phenol-storing cells were recorded at 3 locations within leaf tissues: within the adaxially-located palisade tissue, above the abaxial epidermis and in vicinity of vascular bundles. Dimensions of the all three types were increased in response to infection with Ae5, decreased in response to MDCA treatment and nearly resume the dimensions of those in control leaves in response to the combined treatment. Among the three types of phenol cells, the number of only bundle-associated phenol cells was responded to either Ae5 or MDCA treatment.

INTRODUCTION

Water hyacinth, *Eichhornia crassipes* (Mart.) Solms (Pontederiaceae) continues to pose serious economic, social and environmental problems in Egypt and many other countries in the tropics and subtropics. In recent years, the attention has centered on biological control whereas it could provide a cost-effective, environmentally safe solution to water hyacinth problem. The most widely adopted biocontrol agents are the fungal pathogens (Shabana *et al.*, 1994a, b; Vincent 2001).However,a multicomponent, integrated control approach rather than a single control tactic offers the best prospect for long-term management of aquatic weeds. There are a number of ways to integrate aquatic weed control agents. Integration of pathogens with low doses of chemical herbicides or biochemicals that can interfere with the weed's defence mechanism is also invisioned (Charudattan,1999).In this respect, the potential to exploit synergistic interactions between a plant pathogen and an enzyme inhibitor has been promoted as an option. Trans-cinnamic acid is a potent inhibitor of PAL, phenylalanine ammonia lyase, (Godoy-Hernandez et al,2000) which is the first enzyme in the phenylpropanoid pathway through which plant phenolics are synthesized (Razal et al,1996). Theoretically, this will result in weakness of the weed defense system and therefore the weed becomes more susceptible to the pathogen. The specific objective of the present study is to evaluate the feasibility of integration of a fungal pathogen, *Alternaria*

eichhorniae 5 (Ae5), indigenous to Egypt and host specific to water hyacinth, and the enzyme inhibitor, 3, 4-methylenedioxy trans-cinnamic a cid (MDCA) for the management of water hyacinth.

MATERIALS AND METHODS

Source of fungal culture used

Alternaria eichhorniae Nag Raj & Ponnappa, isolate Ae5, isolated from diseased water hyacinth plants collected in Dakahlia Governorate, Egypt, was used.Working cultures of this isolate maintained in soil tubes were used.

Preparation of Ae5 formulation in oil emulsion

The oil phase consisted of 25% (v/v) soybean lecithin (as an emulsifying agent) and 75% (v/v) cottonseed oil. Lecithin was added to the oil and homogenized in a blender. The aqueous phase contained 1% (w/v) sodium alginate, 0.5% corn syrup, and 0.02% Tween 80. The aqueous phase was added to the oil phase (2 : 3) and mixed in a mixer. Lime [$Ca(OH)_2$] at 0.5% (w/v) and blended Ae5 mycelia were added to the final emulsion to give a final concentration of the fungal propagules of 10% (wet w/v) and mixed well on a stirrer to insure homogeneity.

Laboratory Assay

Healthy-looking, medium-size, 4- to 6-leaf water hyacinth plants were collected from natural infestations around El-Mansoura, Egypt, and acclimated in a greenhouse prior to their use in this study. Experiments were conducted on a bench in a laboratory (25 ±3 C, about 54% relative humidity and approximately 270 $\mu E.m^{-1}.s^{-1}$ light at midday). Individual plants were placed in plastic pots (9-cm diameter, 12-cm depth) containing 400 ml of tap water a nd s ettled for 24 h before treatment. Plants were sprayed with the enzyme inhibitor, 3, 4-methylenedioxy trans-cinnamic acid (MDCA) at 2 concentrations (1 and 2 mM), 12 days and/or 5 h before inoculation with Ae5 formulated in cottonseed oil emulsion. Plants were received 23 ml of MDCA (at each concentration) per plant. Control plants were sprayed with the fungus-free aqueous carrier, MDCA at 1 mM only, MDCA at 2 mM only, or Ae5 in cottonseed emulsion only. Five replicates were made for each treatment and plants were arranged in a completely randomized design. Eight days after inoculation with the fungal formulation, the plants were rated for disease incidence (DI) and disease severity (DS), the former as percentage of infected leaves on the plant and the latter as the percent severity of disease damage. The data were analyzed with the Statistical Analysis System (SAS Institute, 1996). All multiple c omparisons w ere f irst subjected to analysis of variance (ANOVA). Significant differences among treatment means were determined with Duncan's multiple range test.

Further investigation

According to the results obtained from the laboratory test, a second trial was initiated for confirmation of the results obtained. In the second experiment, only the concentration of 2 mM of MDCA was chosen to be applied 10 days before inoculation with the fungal formulation. Water hyacinth plants were prepared as described above. Total phenols were quantified and anatomical examinations were done to determine the number, type, and distribution of the phenol cells in water hyacinth leaves received different treatments.

Determination of the total phenol content

Water hyacinth leaf samples were collected immediately before treatment application (denoted from here on as "at application") and/or 5 days after inoculation (denoted from here on as "after application"). Total phenols' concentration in water hyacinth leaves was determined spectrophotometrically using folin-Ciocalteau reagent at wavelength 650 nm (Sadasivam and Manickam, 1996), and expressed as mg phenol per g fresh weight. The data of total phenol content were analyzed with the SAS (1996). All multiple comparisons were first subjected to ANOVA. Significant differences among treatment means were determined with Duncan's multiple range test.

Anatomical measurements

Leaves were collected 5 days after application of either the fungal formulation or the MDCA. Sections were made in 1-cm^2 leaf blade specimens taken from center, right and left portions of each of four leaves taken from 4 different plants. In all cases, the 3rd leaf from plant top was chosen for anatomical investigation. From each specimen, 3 randomly selected sections were measured. The number of the three types of phenol cells (PCs) was recorded in a 1000-µm-distance in a cross section (CS), and the cell dimensions were measured using calibrated eyepiece micrometer. Within each type of phenol cells, the dimensions of at least 118 cells were measured. Due to the isodiametric nature of the bundle-associated phenol cells, only the diameter was recorded. The data were analyzed with the Statistical Analysis System (SAS Institute, 1996). All multiple comparisons were first subjected to ANOVA. Significant differences among treatment means were determined with Duncan's multiple range test.

RESULTS AND DISCUSSION

Laboratory Assay

Results showed that the efficacy of the fungus Ae5 formulated in cottonseed oil emulsion has been enhanced when applied to water hyacinth pretreated with the MDCA. The highest level of DS was obtained on water hyacinth plants treated with both MDCA (2 mM, either 5 h or 12 days before inoculation with Ae5 formulation) and the fungal formulation (Table 1, Fig. 1). These results are on line with those of Charudattan (1986) where water hyacinth plants were damaged more by the combined treatment of *Cercospora rodmanii* with either 2,4-D amine or diquat compared with treatment by the fungus only.

Table 1: Effect of Ae5 oil emulsion formulation and the enzyme inhibitor, MDCA, used singly or in combinations, on water hyacinth plants.

Treatment[a]	Disease Incidence (%)	Disease severity (%)
1	0 d[b]	0 e
2	100 a	76 c
3	65 c	29 d
4	80 b	37 d
5	100 a	79 bc
6	100 a	82 bc
7	100 a	91 ab
8	100 a	100 a

[a] 1 = Control [no fungus, no enzyme inhibitor (MDCA)]; 2 = Ae5 only (in cottonseed emulsion); 3 = MDCA (1 mM) only; 4 = MDCA (2 mM) only; 5 = MDCA (1 mM) + Ae5, MDCA was sprayed onto water hyacinth plants 5 h before inoculation with the fungal formulation; 6 = MDCA (1 mM) + Ae5, MDCA was sprayed onto water hyacinth plants 12 days before inoculation with the fungal formulation; 7 = MDCA (2 mM) + Ae5, MDCA was sprayed onto water hyacinth plants 5 h before inoculation with the fungal formulation; and 8 = MDCA (2 mM) + Ae5, MDCA was sprayed onto water hyacinth plants 12 days before inoculation with the fungal formulation.

[b] Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Total phenol concentration

Leaf samples of water hyacinth, immediately before the treatment application, contained 0.45 – 0.52 mg total phenols/g fresh weight. However, 5-days after application, the total phenols were slightly increased in the control plants, pronouncedly increased in Ae5-treated plants but decreased in MDCA-treated plants (Table 2). The MDCA cut down the total phenol concentration by 52.0% at the 5th day after application. On the other hand, the total phenols in the leaves treated with both MDCA and Ae5 were nearly equal to that recorded in the untreated plants.

Table 2: Total phenol concentration (mg/g fresh wt) in water hyacinth leaves directly before (at application) and 5 days after (after application) treatment with either MDCA, Ae5, or both compared with those in untreated control plants.

Treatment	At application	After application	± %
Control	0.45 a[a]	0.52 b	+ 15.5
MDCA	0.50 a	0.24 c	- 52.0
Ae5	0.48 a	0.74 a	+ 54.1
MDCA+ Ae5	0.52 a	0.48 b	- 7.6

[a] Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P = 0.05$).

In accordance with the results of the present investigation, it has been reported that Ae5 infection led to an increase in the total phenol content in water hyacinth leaves (Shabana *et al.*, 1997). The accumulation of phenols was considered as a plant defense mechanism not only against fungal pathogens (Martyn and Freeman, 1978; Cody and Martyn, 1979;

Martyn and Cody, 1983), but also against other types of environmental stress (Kumar and Rana, 1994; Wang and Zhang, 1995). According to Martyn and Freeman (1978), *Acremonium zonatum* hyphae which penetrated the phenol cells in water hyacinth leaves appeared vesiculated and nonviable, and that the phenol bodies within the phenol cells tended to accumulate around the invading hyphae.

The application of MDCA decreased the total phenol concentration in water hyacinth leaves, thus weakening the plant defense against Ae5 attack. This may be due to the inhibiting effect of trans-cinnamic acid on the activity of PAL, phenylalanine ammonia lyase (Bolwell *et al.*, 1986; Godoy-Hernandez and Loyola-Vargas, 1991; Godoy-Hernandez *et al.*, 2000). PAL is the first enzyme in the phenylpropanoid pathway through which plant phenolics are synthesized (Razal *et al.*, 1996; Godoy-Hernandez *et al.*, 2000). This conclusion is substantiated by some investigations the results of which showed that other PAL inhibitors such as AOPP (Duke *et al.*, 1979) and 2-AIP (El-Mergawi, 1997) decreased plant phenol content in soybean seedlings and *Catharanthus roseus* leaves, respectively.

Anatomical investigations

1. Spatial distribution of phenol cells within leaf tissues of water hyacinth:

Phenol cells (PCs) were detected in 3 locations within leaf tissues: within the adaxial palisade tissue (Palisade tissue-included phenol cells; PTPCs), directly above the abaxial epidermis (abaxial hypodermal phenol cells; AHPCs), and within the spongy mesophyll, usually in the vicinity of vascular bundler (Fig. 2). PTPCs are much- elongated palisade cells with bigger diameter compared with the chloroplast-rich cells of the palisade tissue, and extending from the adaxial epidermis down, mostly, to the vascular bundle region. AHPCs are palisade-like whereas PCs in the vicinity of vascular bundles are more or less, isodiametric (isodiametric phenol cells, IPCs) (Fig. 2). Maximum, minimum and mean number as well as dimensions of the three types of PCs are presented in Table 3.

Table 3: Maximum, minimum and mean number as well as dimensions of PTPCs, AHPLs and IPCs in untreated control leaves of water hyacinth (healthy leaf).

	Number[a]			Dimensions (μ)					
				Length			Width		
	Max.	Min.	Mean	Max.	Min.	Mean	Max	Min.	Mean
PTPCs	12	5	7.1	138.3	65.5	101.2	36.4	14.6	25.7
AHPCs	6	2	3.2	63.7	25.5	43.6	32.8	12.7	21.9
				Diameter (μ)					
IPCs	5	1	2.4	29.1	12.4	21.5	-	-	-

[a] The number was recorded in a cross section 1000-μm-distance.

Check	MDCA 1 mM Only	MDCA 2 mM Only	Ae5 Only	MDCA 1 mM 5 h before inoculation + Ae5	MDCA 1 mM 12 days before inoculation + Ae5	MDCA 2 mM 5 h before inoculation + Ae5	MDCA 2 mM 12 days before inoculation + Ae5

Fig. 1. Effect of using Ae5 oil emulsion formulation and an enzyme inhibitor (MDCA) singly or in combinations on water hyacinth plants.

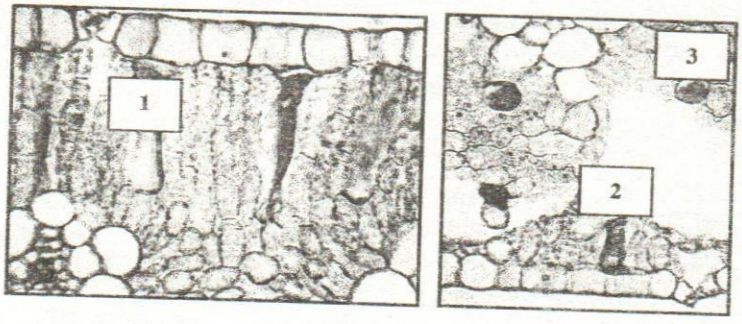

Fig. 2. Phenol cell types in water hyacinth leaf: palisade tissue-included phenol cells (PTPCs, 1), abaxial hypodermal phenol cells (AHPCs, 2), and isodiametric phenol cells (IPCs, 3).

Effects of treatments on the number and dimensions of PCs:

Treatment with Ae5 increased whereas MDCA decreased the dimensions of all types of PCs (Table 4, Fig. 3). The dimensions of PCs in the leaf tissues of plants treated with both Ae5 and MDCA were, more or less, analogous to those in the control plants. On the other hand, the number of PTPCs and AHPCs did not affected by either Ae5, MDCA or both, whereas that of IPCs was increased by Ae5 and decreased by MDCA and the combination Ae5-MDCA.

Table 4: The number[a] and dimensions (μ) of the three types of phenol cells in water hyacinth leaves as affected by either Ae5, MDCA or both compared with the untreated control.

Treatment	PTPCs			AHPCs			IPCs	
	Number	Length	Width	Number	Length	Width	Number	Diameter
Control	6.9 a[b]	98.4 b	25.0 b	3.3 a	40.8 b	22.3 b	2.5 b	22.0 b
MDCA	6.7 a	68.0 c	17.0 c	3.3 a	26.6 c	15.8 c	1.6 bc	15.2 c
Ae5	7.0 a	128.5 a	32.4 a	3.5 a	58.2 a	30.0 a	3.2 a	27.4 a
MDCA+Ae5	7.1 a	102.4 b	23.7 b	3.4 a	38.7 b	20.9 b	2.0 bc	21.0 b

[a] The number was recorded in a cross section 1000-μm-distance.
[b] Values within a column followed by the same letter(s) are not significantly different according to Duncan's multiple range test ($P = 0.05$).

The distribution of PCs in water hyacinth leaf tissues recorded in the present study is comparable to that reported elsewhere (Martyn and Cody, 1983; Martyn *et al.*, 1983). According to Martyn *et al.* (1983), phenolic compounds in water hyacinth leaves are localised in specialized idioplasts (Phenol-storing cells) on both sides of the leaf and also in close association with the vascular bundles, and that those associated with vascular bundles are nearly isodiametric.

Fig. 3. Differential PTPCs dimensions as a response to Ae5 (middle) and MDCA (right) as compared with the untreated control (left).

The unchanged number of PTPCs and AHPCs in response to the different treatments may imply the differentiation of these two types of PCs early in leaf developmental growth. Hence, the plant response to an environmental stress may be mediated through increasing synthesis and storage of phenolic compounds inside these previously-formed cells, consequently increasing their dimensions. On the other hand, the changing number of IPCs in response to the applied treatments may imply that these are unspecialized phenol-storing cells and their differentiation is a growth condition-dependent.

It could be concluded that water hyacinth plants pretreated with MDCA are more susceptible to Ae5 due to weakness of their phenolic defense mechanism.

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المقاومة المتكاملة لورد النيل باستخدام الفطر *Alternaria eichhorniae* (Ae5) ومثبط إنزيمى لمسار تخليق الفينولات فى النبات

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يعتبر الفطر *Alternaria eichhorniae* (المعزول من البيئة المصرية والمتخصص على وردالنيل) عامل مقاومة حيوية ذو كفاءة عالية فى مقاومة ورد النيل فى مصر. وقداستخدم هذا الفطر فى الدراسة الحالية فى صورة مبيد حيوى مجهز فى مستحلب زيتى من زيت بذرة القطن والذى أثبت كفاءة عالية فى مقاومة ورد النيل فى ظروف الحقل. وكان الهدفالرئيسى للبحث الحالى هو دراسة إمكانية زيادة كفاءة المبيد الحيوى على ورد النيل عن طريق إضعاف الجهاز المناعى للنبات بشل قدرته على تخليق المركبات الفينولية (التى يفرزها النبات عادة لمقاومة فعل المسببات المرضية كوسيلة للدفاع عن نفسه) وذلك بمعاملة النبات بالمثبط الإنزيمى ميثيلين ديوكسى ترانس سينامياك أسيد (MDCA) الذي يثبط الإنزيم فينايل ألانين أمونيا لاييز (PAL) وهو المسئول عن تخليق المركبات الفينولية فى النبات. وقد أثبتت نتائج التجارب التى أجريت فى هذا البحث نجاح هذه النظرية حيث حدث موت لنباتات ورد النيل المعاملة بالمثبط الإنزيمى والمبيد الحيوى معا فى وقت أقصر بالمقارنة بالنباتات المعاملة بالمبيد الحيوى فقط (بمفرده).

ولقد أوضحت النتائج أن العدوى بالفطر Ae5 سببت زيادة التركيز الكلى للفينولات بينما المعاملة بمادة MDCA قد أدت الى نقصها فى أوراق نباتات ورد النيل. ولقد كان مستوى تركيز الفينولات الكلية فى النباتات التى عوملت بكليهما مقاربا لنظيره فى النباتات الغير معاملة. ومن الناحية التشريحية، وجدت الخلايا المخزنة للفينولات فى ثلاث مواقع داخل أنسجة الورقة: داخل النسيج العمادى الموجود أسفل البشرة العليا ، ومباشرة أعلى البشرة السفلى ، وبجوار الحزم الوعائية للورقة. ولقد زادت أبعاد الثلاث طرز من الخلايا المخزنة للفينولات استجابة للعدوى بفطر Ae5 بينما نقصت استجابة للمعاملة بمادة MDCA. وكانت أبعاد تلك الخلايا فى أوراق النباتات المعاملة بكليهما مقاربة لنظائرها فى النباتات الغير معاملة. وفيما يتعلق بعدد الخلايا الخازنة للفينولات ، كان عدد تلك المجاورة للحزم الوعائية فقط هو الذى تأثر تبعا لنوع المعاملة.