Turkish Journal of Agriculture and Forestry

Volume 26 | Number 6 Article 6

1-1-2002

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Turk J Agric For
26 (2002) 343-348

Induction of Phytoalexin Accumulation in Broad Bean (*Vicia faba* L.) Cotyledons Following Treatments with Biotic and Abiotic Elicitors

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Received: 01.03.2002

Abstract: Broad bean (*Vicia faba*) cotyledons that were inoculated artificially with fungal pathogens or exposed to various abiotic agents were analysed for phytoalexin production. Biotic elicitors, such as *Botrytis cinerea* and *B. allii*, and abiotic elicitors, such as ultraviolet (UV) radiation (254 nm) and freezing-thawing, were used to induce phytoalexin accumulation. Wyerone and other wyerone derivatives were the major phytoalexins responding in broad bean cotyledons. The quantities of wyerone within elicitor-treated tissues were examined by thin layer chromatography. The highest amount of wyerone was induced by *B. cinerea* (943 µg/g fresh weight). Treatment of cotyledons with UV radiation (452 µg/g f.wt), *B. alli* (325 µg/g f.wt) and freezing-thawing (288 µg/g f.wt) also caused considerable activation of the phytoalexin synthesis. Cell necrosis and wyerone accumulation were closely associated, and the highest concentration of wyerone was in tissue bearing brown lesions. Only very low concentrations of wyerone accumulated at sites of mechanical damage. The results indicate that the presence of both damaged and healthy tissues is necessary for phytoalexin production.

Key Words: Broad bean, *Vicia faba*, phytoalexin, induced resistance, abiotic elicitors

Biyotik ve Abiyotik Uyarıcılar ile Muamele Edilen Bakla Kotiledonlarında (*Vicia faba* L.) Fitoaleksin Birikiminin Teşvik Edilmesi

Özet: Fungal patojenlerle yapay olarak inokule edilen veya çeşitli abiyotik etkenlere maruz bırakılan bakla kotiledonlarında fitoaleksin üretimleri analiz edilmiştir. *Botrytis cinerea* ve *B. allii* biyotik elisitörler olarak, ultraviole radyasyonu ve dokuların dondurulup-çözülmesi uygulamaları ise abiyotik elisitörler olarak fitoaleksin birikimini teşvik etmekte kullanılmıştır. Wyerone ve diğer wyerone türevleri bakla kotiledonlarında ortaya çıkan önemli fitoaleksinlerdir. Elisitörler ile muamele edilmiş bakla kotiledonlarında ki wyerone miktarları ince tabaka kromotografi (TLC) kullanılarak incelenmiştir. En yüksek wyerone miktarı *B. cinerea* (943 µg/g taze ağırlık) tarafından teşvik edilmiştir. Kotiledonların UV uygulaması (452 µg/g f.wt), *B. allii* uygulaması (325 µg/g f.wt) ve dondurup-çözme uygulamaları (288 µg/g f.wt) ile muamele edilmeleri de fitoaleksin tepkimesinin ortaya çıkmasına sebep olmuştur. Hücre nekrozlaşması ile wyerone birikimi arasında yakın bir ilişki söz konusu olup, en yüksek wyerone konsantrasyonu sınırlı lezyonların oluştuğu kahverengileşmiş dokularda oluşmuştur. Mekanik zararlanmaların yapıldığı noktalarda çok düşük konsantrasyonlarda wyerone birikimleri tespit edilmiştir. Sonuçlar fitoaleksin üretimi için zarar görmüş ve sağlıklı dokuların birlikte bulunması gerektiğini göstermektedir.

Anahtar Sözcükler: Bakla, *Vicia faba*, fitoaleksin, dayanıklılığın teşviki, abiyotik elisitör

Introduction

Plants have evolved very sophisticated physical and biochemical mechanisms against pathogen infections. The mechanisms of plant response to infection occur at both cellular and subcellular levels and involve co-ordinated events that begin soon after parasite encounter. Examples of the defence mechanisms include the accumulation of anti-microbial compounds, the fortification of cell wall structure, induction of hydrolytic enzymes and other defence-related proteins and activation of the hypersensitive reaction (Hammerschmidt and Schultz, 1996, Hammond-Kosack and Jones, 1996). The physiological and biochemical basis of plants resistance to fungal, bacterial and viral pathogens has been associated with both preformed and infection-induced antimicrobial compounds (Osbourn, 1996). One

of the best and extensively-studied defence responses of plants to pathogen infection is the induced accumulation of secondary metabolites such as phytoalexins (Hammerschmidt, 1999). Phytoalexins are a diverse group of low molecular weight anti-microbial compounds that are synthesized and accumulated in appreciable amounts in plants after stimulation by the various types of pathogens or by chemical or mechanical injury and are toxic to pathogens (Smith 1996, Mansfield, 1999).

The production of phytoalexins after infection suggests that a product of the pathogen or host-pathogen interaction is involved in triggering phytoalexin biosynthesis. A variety of pathogen and plant-produced molecules, collectively known as biotic or abiotic elicitors, induce phytoalexins and other defence responses (Yoshikawa et al., 1993; Hahn, 1996). Biotic elicitors originate from the pathogen or aggressors themselves and evoke a response in cells in the immediate vicinity of the pathogen. Abiotic elicitors include chemicals, such as mycoherbicide, mercury salts and copper, physical agents such as injury, partial freezing and thawing, and UV radiation (Sharon and Gressel, 1991, Canıhoş et al., 2000, Hanawa et al., 2000, Lamikanra et al., 2002). Tissues of *V. faba* respond to infection with *Botrytis* by the production of five furano-acetylenic compounds possessing antifungal activity. Wyerone acid was the major phytoalexin recovered from leaves and pods. By contrast, wyerone was by far the predominant phytoalexin reported in cotyledons (Hargreaves et al., 1977).

The objective of this study was to determine the potential of broad bean cotyledons to synthesize and accumulate a range of phytoalexin in response to biotic and abiotic elicitors. A secondary question was whether formation of phytoalexin was a specific response to pathogen or a general reaction to stress factors. Therefore, two abiotic elicitors (short-wave UV irradiation and a cycle of freezing-thawing) were included to compare their elicitor activity with that of the pathogens. The relative effectiveness was compared of both biotic and abiotic elicitors in causing the accumulation of wyerone in cotyledons.

Materials and Methods

Cotyledons were obtained from undressed seeds of broad bean plants (*Vicia faba* L.), cv Sutton. *B. cinerea* and *B. allii* were isolated from infested bean and onion plants grown at Wye, UK. All fungal species were maintained on potato glucose agar (PDA, Merck), incubated at 18 ºC with a 16 h photo-period. Spore concentrations were adjusted to 10^5 spores/ml as previously described (Hargreaves et al., 1977).

Phytoalexin Induction

The accumulation of phytoalexins was stimulated in several ways. Each treatment was applied to broad bean cotyledons (n = 15). Imbibed broad bean seeds were peeled and split into two cotyledons. Cotyledons were then put inside plastic sandwich boxes lined with moistened tissue paper to prevent the cotyledons from drying out. The treatments were applied as follows:

1. Inoculation of *B. cinerea* and *B. allii*: 40 µl inoculum droplets containing 10^5 spores /ml were deposited on the cotyledon surfaces.

2. Exposure to UV radiation: The cotyledons, placed in sandwich boxes with their lids open, were exposed at a distance of 10 cm from UV radiation source (254 nm: 180 µW $cm^{-2:}$ CP Instrument Co., UK) for 15 min.

3. Freezing and thawing: One-third of the cotyledons was dipped into liquid nitrogen for 30 s and placed into the sandwich box.

4. Controls: The cotyledons were plated into the sandwich box without any treatment or inoculated with sterile water.

After the application of various treatments, cotyledons in the sandwich boxes were incubated in a growth room maintained at 18 ºC with a 16 h photo-period, for a week to allow possible interaction.

Extraction Procedures and Quantitative Measurement of Wyerone Accumulation

A week after induction with several elicitors, tissues were collected by slicing various layers for phytoalexin extraction. In cotyledons inoculated with *B. cinerea* and *B. allii*, samples were collected from the upper layers (sites inoculated with conidial suspension, not more than 1 mm thick) and underlying tissues. In UV radiation-exposed cotyledons, tissues were collected from the bronzing layer at the surface and the underlying layer as described for *Botrytis*-treated cotyledons. In freezing and thawing treatment, samples were taken from the frozen part, the healthy part and from the interface between the healthy and frozen sites. Tissues collected from water-inoculated

or non-inoculated cotyledons were used as controls. For quantitative analyses, samples of treated tissues (1-2 g fresh weight) were excised and subsequently homogenized in 100% methanol with a Polytron blender until a fine suspension was obtained. The samples were then centrifuged at 4000 g for 10 min and transferred to a clean tube. The pellet was re-extracted with a further 5 ml of 100% methanol and both supernatants were then combined and dried in vacuo at 30 ºC.

The methanolic soluble residue was dissolved in a small amount of methanol (0.5 g fwt/150µl) and applied to 5 cm origins on precoated thin layer chromatography (TLC) plates (Merck Kieselgel 60 F254 Silica gel). Plates then were developed in tanks pre-equilibrated with chloroform : ethanol (98:2, v/v), air dried and assessed. Spots were detected on TLC plates by their characteristic appearance UV light (366 nm). The phytoalexin, wyerone, was detected on TLC plates by its blue-purple fluorescence under UV with an R*f* value of c. 0.6. Fluorescing bands containing wyerone were located on the TLC plates and then scraped off the plate and eluted in 1 ml of 100% methanol. Wyerone has a characteristic spectrum with λ_{max} at 351 nm. The amounts of wyerone in tissues based on UV spectra were calculated using a molar extinction coefficient of Fawcett et al. (1969).

TLC plate bioassays were used to detect antimicrobial compounds in extracts (Bennett et al., 1994). Methanolic extracts of inoculated tissue were applied to 5 cm origins on pre-coated TLC plates. Following development of chromatograms, plates were air-dried and sprayed with concentrated suspensions (5 x 10^8 spores/ml) of conidia of *Cladosporium herbarum* in potato glucose solution. The sprayed plates were incubated at 21 ºC in darkness in a moist chamber for 4 days. The fungus typically failed to grow where antimicrobial compounds were present. Zones inhibitory to *C. herbarum* were recognized as white areas of silica gel where the dark green fungus failed to grow.

Results presented were means for three separate experiments in which each treatment was replicated three times with different lots of cotyledons (1 g fresh weight each). Mean values were subjected to analysis of variance (one-way ANOVA), and the Duncan's multiple range test (SPSS statistical package, 10.0) was used to determine significant differences ($p \leq 0.01$) between treatments.

Results

Symptoms on Cotyledons

Both *B. cinerea* and *B. allii* caused limited lesions on bean cotyledons. Lesions produced by *B. cinerea* were orange or brick red. Lesions produced by *B. allii* were lighter than those produced by *B. cinerea*. These lesions were confined to the tissue beneath inoculum droplets. Cotyledons exposed to UV radiation developed a bronzing of the surface. Cotyledons receiving the freezing and thawing treatment produced three district symptoms: (i) the frozen part was dead and some parts colonized by saprophytic fungi and bacteria; (ii) the unfrozen part was apparently healthy and (iii) the border between the healthy and frozen area was pigmented. Non-inoculated cotyledons seemed almost healthy in appearance. Some cotyledons inoculated with sterile distilled water were slightly rotted in places due to high moisture in the storage boxes.

The presence of antifungal compounds in cotyledon tissues was clearly observed with bioassay. A typical TLC plate bioassay clearly indicates that a number of antimicrobial compounds were detected by this technique (Figure 1). The identification and antifungal activity of the five major bands was associated with a compound which fluoresced bright blue-purple under UV radiation as previously reported for wyerone, wyerone epoxide, wyerol, medicarpin and wyerone acid (Hargreaves et al., 1977).

In repeated experiments, the distribution of phytoalexin within treated and underlying tissues of cotyledon inoculated with *Botrytis* spp. or exposed to UV showed that wyerone, wyerone epoxide and wyerol were confined to the treated sites (Figure 1).

Changes in Wyerone Accumulation in Tissue Treated with Different Elicitors

The amount of wyerone produced by the broad bean cotyledons in response to various elicitors is presented in Table 1. In all treatments, the accumulation of wyerone was associated with the appearance of symptoms. Both biotic elicitors, *B. cinerea* and *B. allii*, induced high levels of wyerone. The accumulation of wyerone closely reflected the number of dead cells at reaction sites. Limited lesions caused by *B. cinerea* were larger than those produced by *B. allii*. Thus the amount of wyerone in tissue inoculated with *B. cinerea* was higher than recorded in tissue inoculated with *B. allii* (Table 1). The

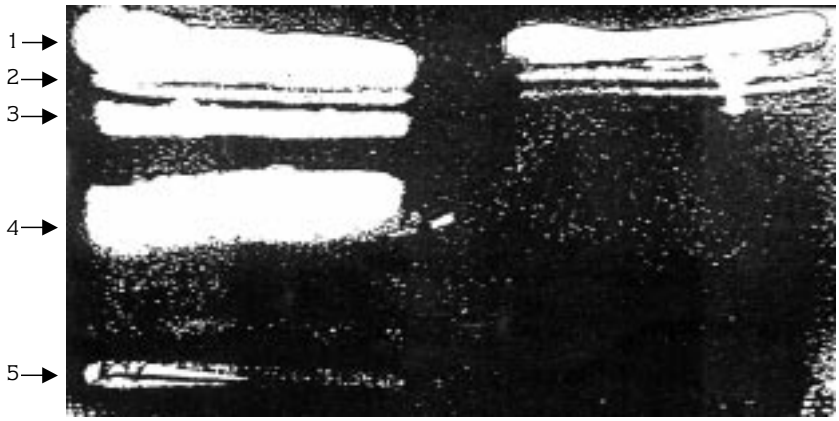

Figure 1. Thin layer chromatography plate bioassay with *Cladosporium herbarum* detects phytoalexins in *cotyledon tissue challenged by B. cinerea*. Tissue collected 7 days after inoculation with *B. cinerea*. Extracts applied to 4 cm origins of lane 1 obtained from treated layer and lane 2 from underneath (untreated) layer. Lane 1 shows 5 distinct bands of wyerone (1), wyerone epoxide (2), wyerol (3), Medicarpin/PA4 (4) and wyerone acid (5), respectively. Note the absence of medicarpin and wyerone acid within the underlying (untreated) layer (lane 2).

Table 1. Accumulation of wyerone[a] within the necrotic lesion (treated), surrounding tissue (untreated) and interface sites at 7 days after challenge with biotic and abiotic agents.

Treatments	Accumulation of wyerone (µg/g f.wt) at different sites[b]		
	Treated	Untreated	Interface
Control [c]	25 C	10 A	N/A[d]
Botrytis cinerea	943 A	4 A	N/A
Botrytis alli	325 B	11 A	N/A
UV (254 nm)	452 B	16 A	N/A
Freeze-thawed	2 Ca	5 Aa	288 b

[a] Amounts of wyerone were quantified by UV absorption spectroscopy as described in the materials and methods section. Values are presented as the means of three independent experiments. Mean values (n = 3) followed by the same capital or small letter within the column or row, respectively, are not significantly different according to Duncan's multiple range test ($p \leq 0.01$).

[b] Samples of treated tissues (1-2 g fresh weight) were excised from the treated layers (sites treated with conidial suspension, bronzing layer at the surface, frozen part or water-treated and not more than 1 mm thick) and untreated layer (sites beneath the necrotic layer about 1 mm thick which included apparently healthy tissue). The interface site is the area between healthy and frozen sites, see materials and methods for details.

[c] Tissues collected from water-inoculated (treated) or non-inoculated (untreated) cotyledons were used as controls.

[d] N/A = Not applicable.

distribution of wyerone within the inoculated site (lesion) and in the surrounding site (about 1 mm thick which included apparently healthy tissue) was also examined. The highest concentrations of wyerone were consistently found in collapsed brown tissue at inoculation sites. Very low levels of wyerone were detected in tissue underlying the upper layer (underneath layer). UV radiation also induced a substantial amount of wyerone, i.e. 452 mg/g f.wt. In frozen-thawed tissue, the highest amount of wyerone was recovered within the mixture of dead and live cells at the interface (Table 1). Very low levels of wyerone were produced by tissues in either the frozen dead cells or the healthy live cells.

Small amounts of wyerone were found in control treatments. The amount of wyerone was higher in water-inoculated cotyledons than those in non-inoculated cotyledon (Table 1). No phytoalexin was recovered from the totally rotten tissues.

Discussion

Phytoalexin accumulation is believed to be an important early defence mechanism in several plant-pathogen interactions, and it is known that at least five phytoalexins are produced by *Vicia faba* in response to infection with *Botrytis* spp. (Hargreaves et al., 1977). In our study, broad bean cotyledons responded to biotic and abiotic elicitors by the production of five different phytoalexins possessing antifungal activity, wyerone and wyerone derivatives. Although phytoalexins are elicited by biotic agents such as plant pathogenic fungi, their presence complicates the results since not only may they elicit phytoalexins but may also degrade them (VanEtten et al., 1995; Sobby et al., 1996). This problem can be circumvented by the use of physical or chemical abiotic elicitors. Zahringer et al. (1979) have indicated that biotic, but not abiotic elicitors cause the accumulation of glyceollin in soybean tissue. Our results, however, clearly demonstrate that abiotic elicitors are as good as biotic elicitors in the induction of considerable amounts of phytoalexins in broad bean cotyledon. Similar results were also reported in several plants when comparing the efficiency of abiotic elicitors to that of fungal pathogen in inducing phytoalexins (Subba Rao et al., 1996; Marley and Hillocks, 2002).

The resistance of several plants to their potential pathogen has recently been associated with the localized production of phytoalexin in and around the tissue (Cooper et al., 1996). In our study, the highest concentration of wyerone was found in brown tissue within the site of inoculation where fungal growth was restricted. Tissue dissected from the underlying layer contained considerably lower amounts of wyerone. Initially, phytoalexins were hypothesized to be involved in the cause of cell death during the hypersensitive reaction (VanEtten and Bateman, 1971). In beans, phaseollin accumulated rapidly following the death of cells during incompatible interaction. The highest yields of phaseollin were recovered from excised inoculation sites bearing limited lesions (Soriano-Richards et al., 1998). The patterns of wyerone accumulation detected in our study support the proposal that cell death precedes major accumulation of phytoalexins within infected cells (Bailey et al., 1980, Bennett et al., 1994) or surrounding tissue. The localization of phytoalexins, wyerone and wyerone acid in the infected and living host cells in broad bean leaves infected with *Botrytis cinerea* was previously determined by microspectrophotometry (Mansfield et al., 1974). The presence of the wyerone in live cells in cotyledon tissue could be the result of its production by these cells or accumulation from adjacent necrotic cells and the surrounding intercellular fluid. Totally rotten cells, however, failed to accumulate any antimicrobial compounds, which may either emphasize the role of healthy cells in the production of these compounds, or phytoalexin produced in rotten tissue could have been metabolized by saprophytic organisms.

From the above results, it is apparent that limited lesion-bearing cotyledon inoculated with *B. cinerea* produced a high amount of wyerone. Hargreaves et al. (1977) showed that the rapid accumulation of wyerone could alone account for the restriction of fungal growth in cotyledons. A close correlation between the accumulation of phytoalexin and the restriction of microbial growth was also reported in lettuce and groundnuts (Bennett et al., 1994, Subba Rao et al., 1996).

The presence of effective elicitors is very often associated with the death of and injury to plant tissue. Healthy plant cells contain materials capable of initiating the synthesis of appropriate enzymes within the cell but these usually remain inactive until the introduction of an appropriate elicitor. Rahe and Arnold (1975) reported that small freeze-injury sites on hypocotyls of *Phaseolus vulgaris* seedlings accumulated significant levels of phaseollin. Studies by Hargreaves and Bailey (1978) showed that aqueous extracts of French bean hypocotyls, which had been frozen in liquid nitrogen, stimulated phytoalexin phaseollin production. Coupling these findings with the evidence for endogenous elicitors suggests that the production of phytoalexin in *Vicia faba* tissue following exposure to abiotic elicitors could be due to the release of a plant's constitutive elicitor from the damaged cells.

In conclusion, we have indicated that there are probably constitutive phytoalexin elicitors in healthy broad bean tissue which induce significant amounts of phytoalexins. The possible contribution of abiotic elicitors, such as UV treatments, in the production of phytoalexins, which are implicated in resistance to microbial pathogen, should not be overlooked. Further work is, however, required to isolate and characterize this elicitor and to confirm its role in the bean's resistance to its potential pathogens. Detection of very low amounts of wyerone in

the tissue away from the surface at infected sites and failure of completely rotten tissue to accumulate phytoalexins may suggest a requirement for healthy cells for accumulation of phytoalexin. Whether phytoalexins produced in rotten tissue are metabolized by the saprophytic organisms should be further investigated.

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