Inducible Enzymes of the 9,10-Dihydro-phenanthrene Pathway. Sterile Orchid Plants Responding to Fungal Infection

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Bulbs, roots, and rhizomes of orchids may contain substantial amounts of 9,10-dihydrophenanthrenes being formed from bibenzyls in an oxidative coupling reaction. They are, therefore, products of the phenylpropane metabolism in combination with polyketide formation. In young sterile plants of *Phalaenopsis* species, however, these compounds were not detectable. Infection by *Botrytis cinerea* and *Rhizoctonia* spp. caused a pronounced increase in the amount and enzyme activity of bibenzyl synthase, the key enzyme of the pathway. Concomitantly, phenylalanine ammonia-lyase and an *O*-methyltransferase were induced. 3,5-Dihydroxy-3′-methoxybibenzyl and the phytoalexin hircinol (2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene) were formed. Infection of leaves of young orchid plants with *Botrytis cinerea* led to a more than 100-fold increase in bibenzyl synthase activity within one day. Using an antiserum raised against a bibenzyl synthase from *Bletilla striata* we studied the change of bibenzyl synthase protein during the time following elicitation. Roots infected with *Rhizoctonia* sp. showed a similar but less rapid response.

Mycorrhiza and incompatible interactions between plants and fungi appear to be associated with the activation of plant genes encoding phytoalexin-forming enzymes. In the case of an attack of pathogenic fungi, the plant often reacts by an intensive but transient response (Dixon and Lamb 1990). Instances of symbiotic relationships indicated that the plant reaction upon the presence of the fungus is markedly reduced (Hartwig and Phillips 1991; Parniske *et al.* 1991). Mycorrhizal fungi are sensitive to certain phytoalexins (Gäumann 1959; Gäumann and Kern 1960). Earlier work with orchid rhizomes housing various amounts of endogenous fungus showed that virtually no phytoalexin was formed during symbiosis but revealed that upon destruction of the mycorrhiza the response of the plant was approximately proportional to the degree of fungal material present (Gehlert and Kindl 1991). Furthermore, it was hypothesized that during establishment of a mycorrhiza the sensor of the plant response mechanism becomes more and more inactivated, leading eventually to a plant stage characterized by a very low level of phytoalexin production.

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In terms of chemical structures, the main phytoalexins of orchids are 9,10-dihydrophenanthrenes. Gäumann (1959), one of the first investigators to develop the concept of phytoalexin, demonstrated that the amount of hircinol and orchinol increased from very low values in healthy tubes to concentrations as high as 3 g/kg tubes in infected tissue. In terms of biosynthesis (Fritzemeier and Kindl 1983; Gehlert and Kindl 1991), dihydrophenanthrenes may be classified as stilbenoids as they are derivatives of dihydrostilbenes (or bibenzyls).

While the pathway to hydroxystilbenes is well established and data on the enzymes (Schöppner and Kindl 1984; Gehlert *et al.* 1990; Liswidowati *et al.* 1991) and the respective cDNA structures (Schröder *et al.* 1988; Melchior and Kindl 1991; Schanz *et al.* 1992; Schwekendiek *et al.* 1992) are available, less is known about the reaction sequence and the key enzymes modulating the pathway leading to bibenzyls (Fritzemeier and Kindl 1983). There are indications that the bibenzyl synthase activity presents the limiting factor in this pathway (Fig. 1). Thus, the amount of bibenzyl synthase formed may be a measure of how intensively the plant recognizes the presence of a fungus. By applying this parameter, we intend to follow the fungus-induced plant reactions during the transition of a sterile plant to an established mycorrhiza. To proceed towards this goal, we started out describing the predominant changes in plant defense biochemistry during the first steps towards symbiosis.

Based on previous work (Reinecke and Kindl 1994) with the bibenzyl synthase from *Bletilla striata* we used the monospecific antibodies against this enzyme as a tool to study intensities and kinetics of plant response. For leaves and roots of young sterile *Phalaenopsis* plants, we show the pronounced formation of bibenzyl synthase and its rapid degradation.

RESULTS

_O_-Methyltransferase and bibenzyl synthase are inducible.

Previous studies with fully differentiated tissues, i.e., roots of *Epipactum palustris* (Gehlert and Kindl 1991) and bulbs of *B. striata* (Fritzemeier and Kindl 1983), have shown that the pathway to dihydrophenanthrenes is inducible in these plants. Therefore, our first approach was to see whether in the case of *Phalaenopsis* species also young and very young sterile plants were able to respond by establishing the dihydrophenanthrene pathway. At 24 hr after infection with *Botrytis cinerea* we investigated the enzymes that are subject

to induced formation. Although the investigations concentrated first on phenylalanine ammonia-lyase (PAL) and bibenzyl synthase, it soon became clear that the extracts from induced plants contain an enzyme modifying the product of the bibenzyl synthase reaction. To obtain an overview, the experiment was done with 6-mo-old plants that had leaves 1.2 cm long. In preliminary experiments, it was found that the response, the induction of the dihydrophenanthrene pathway,

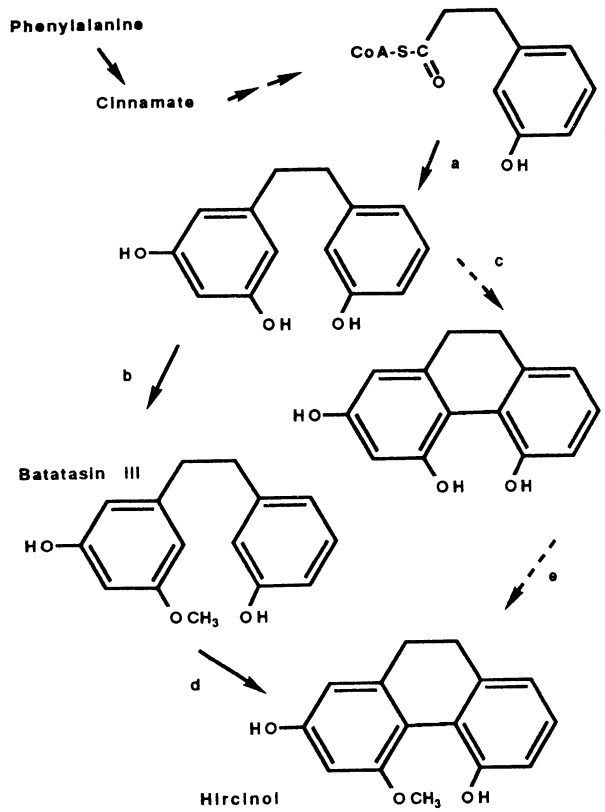

Fig. 1. Alternative pathways leading to the 9,10-dihydrophenanthrene phytoalexin hircinol. Dihydro-*m*-coumaroyl-CoA is converted into 3,3′, 5-trihydroxybibenzyl by the catalysis of bibenzyl synthase (a). From this compound, two pathways (b, d and c, e) lead to the phytoalexin hircinol. On the basis of the experiments reported here, the route via batatasin III is the preferred sequence.

was reasonably high at this stage of seedling growth. A crude extract was prepared at 24 hr after inoculation with *B. cinerea* and passed through a molecular sieve to remove low molecular weight material. In particular cases when we intended to detect consecutive reactions, we abandoned the molecular sieve step. In the infected plant, PAL-specific activity was 0.15 pkat/mg protein compared to less than 0.002 pkat/mg in uninfected tissue. In addition, the separation by TLC of the radioactive products of the bibenzyl synthase assay revealed that, besides trihydroxybibenzyl, an additional peak (X) appeared at a retardation factor value that indicated a compound less hydrophilic than the trihydroxy compound. In relation to the migration of other potential products under the condition of the assay (Fritzemeier and Kindl 1983) and the dependence of its formation from the presence of dihydro-*m*-coumaroyl-CoA and malonyl-CoA, the chromatographic properties of X were in agreement with the presence of a dihydroxy-monomethoxybibenzyl. Following an incubation 20 times as large as the standard assay, the products were separated by TLC, and X characterized by mass spectrometry, indicating a mass of $C_{15}H_{16}O_3$ and a hydroxybenzyl fragment. Table 1 shows that X occurred when crude extracts were employed but was absent in protein fractions passed through molecular sieves. A similar phenomenon was encountered earlier with other plants when methyltransferases were tested in crude extracts (unpublished results). Obviously, this coupled assay of bibenzyl synthase and methyltransferase was effective only when low molecular material was not removed from the crude plant extract.

The radioactive compound X formed *in vitro* was purified chromatographically. Two hundred becquerels of this preparation (with a specific activity of approximately 2 MBq/mmol) were administered to slices of *B. striata* and left there 10 hr for metabolism *in vivo*. The subsequent analysis of the radioactive products showed virtually exclusively hircinol (75% of the radioactivity applied), i.e., 2,5-dihydroxy-4-methoxy-9,10-dihydro-phenanthrene (Fritzemeier and Kindl 1983; Fritzemeier *et al.* 1984). As deducible from Figure 1, compound X was thus identified as 3,3′-dihydroxy-5-methoxybibenzyl, also known as batatasin III (Hashimoto and Hasegawa 1974). Furthermore, Table 1 shows that the formation of labeled dihydroxy-monomethoxybibenzyl was observed when [*methyl*-^{14}C]S-adenosylmethionine was used

Table 1. Formation of trihydroxybibenzyl and dihydroxy-methoxybibenzyl from dihydro-*m*-coumaroyl-CoA and malonyl-CoA

Exp. no.	Plant material (time after infection)	Enzyme preparation	Dihydro-*m*-coumaroyl-CoA	Incubation Malonyl-CoA labeled	SAM exogenous[b]	Products (relative units)[a] Trihydroxy-bibenzyl	Dihydroxy-methoxy-bibenzyl
1	1 h	Crude extract	+	+	−	<1	<1
2	20 h	Crude extract	+	+	−	100	15
3	48 h	Crude extract	+	+	−	25	60
4	20 h	Protein fraction[c]	+	+	−	100	<1
5	48 h	Protein fraction	+	+	−	75	<1
6	20 h	Protein fraction	+	+	+	60	50
7	48 h	Protein fraction	+	+	+	15	60
8	48 h	Protein fraction	−	+	+	<1	<1
9	1 h	Protein fraction	+	Unlabeled	Labeled	<2	<2
10	20 h	Protein fraction	+	Unlabeled	Labeled	ND	50
11	48 h	Protein fraction	+	Unlabeled	Labeled	ND	100

[a] 100 relative units correspond to 0.15 pkat.
[b] S-Adenosylmethionine was either added in non-radioactive form (indicated by "+") or as (*methyl*-^{14}C)-labeled from (indicated by "labeled").
[c] Passed through PD-10 column.

as sole radioactive compound in the incubation mixture.

As a control to assess the selectivity of the orchid methyltransferase, its activity was assayed in a way described previously for caffeoyl-CoA-specific 3-*O*-methyltransferase (Pakusch *et al.* 1989). As phenolic substrate, dihydro-*m*-coumaroyl-CoA was incubated with [*methyl*-^{14}C]-*S*-adenosylmethionine. Virtually no formation of cinnamate derivatives was detectable.

In addition, the data in Table 1 show an almost concomitant increase of both enzyme activities of bibenzyl synthase and *O*-methyltransferase. Controls using non-infected seedlings demonstrated that no significant increase in activities was observed during a 60-hr period.

Infection of *Phalaenopsis* hybrid leaves by *B. cinerea*.

To measure the increase in the level of bibenzyl synthase protein suggesting an activation of its *de novo* synthesis, we applied antibodies for semi-quantitative assays. First, we showed that the antiserum raised against the bibenzyl synthase from *B. striata* was applicable to the analysis of the enzymes from other orchids and was also specific in these cases. Figure 2 demonstrates that the antiserum cross-reacted with bibenzyl synthases from hybrids of *Phalaenopsis* and *Cymbidium*. In addition, Figure 2 shows that the bibenzyl synthase protein was detectable under these conditions only in preparations from infected plants.

We assayed young plants of *Phalaenopsis* hybrids (3 to 30 wk old) during the growth under sterile conditions and found that traces of bibenzyl synthase activity were detectable only with very sensitive methods, i.e., by using incubation mixtures

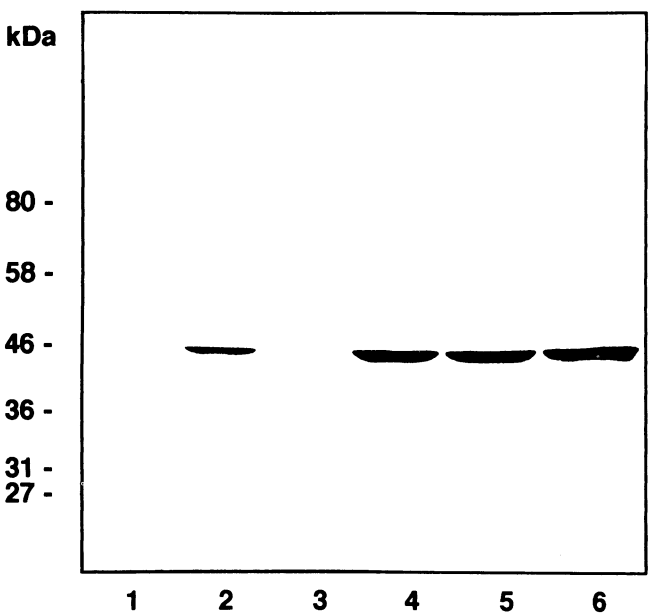

Fig. 2. Anti-bibenzyl synthase antibodies tested as appropriate means for detection of bibenzyl synthase in various orchids. Following electrophoresis and blotting, immunodecoration was performed using antibodies raised against the bibenzyl synthase from *Bletilla striata*. Lane 1, preparation from *Cymbidium* hybrid untreated; lane 2, preparation from *Cymbidium* hybrid treated with *Botrytis cinerea* for 30 hr; lane 3, preparation from *Phalaenopsis* hybrid untreated; lane 4, preparation from *Phalaenopsis* hybrid treated with *Rhizoctonia crocorum* for 40 hr; lanes 5 and 6, two different isoenzymes from *B. striata* separated previously by isoelectric focusing.

10-fold as large as in the standard assay and by utilizing the limits of the detection of radioactivity in the product. Induction of bibenzyl synthase could be brought about, by infection with *B. cinerea*, irrespective of the age of the plants. However, when sterile plants older than 9 mo grown in a greenhouse were analyzed in the same way, the response was significantly lower. Figure 3 shows the profiles of enzyme activities of bibenzyl synthase and PAL during the period after inoculation with *B. cinerea*. Bibenzyl synthase activity was increased by a factor higher than 100. The data demonstrate that the increase of bibenzyl synthase activity and bibenzyl synthase protein was most pronounced between 10–20 hr after onset of infection. Formation of lesions or damage detectable by light microscope did not take place during that time. Thus, the response of *Phalaenopsis* seedlings upon contact with the fungus takes place prior to visible pathogenesis.

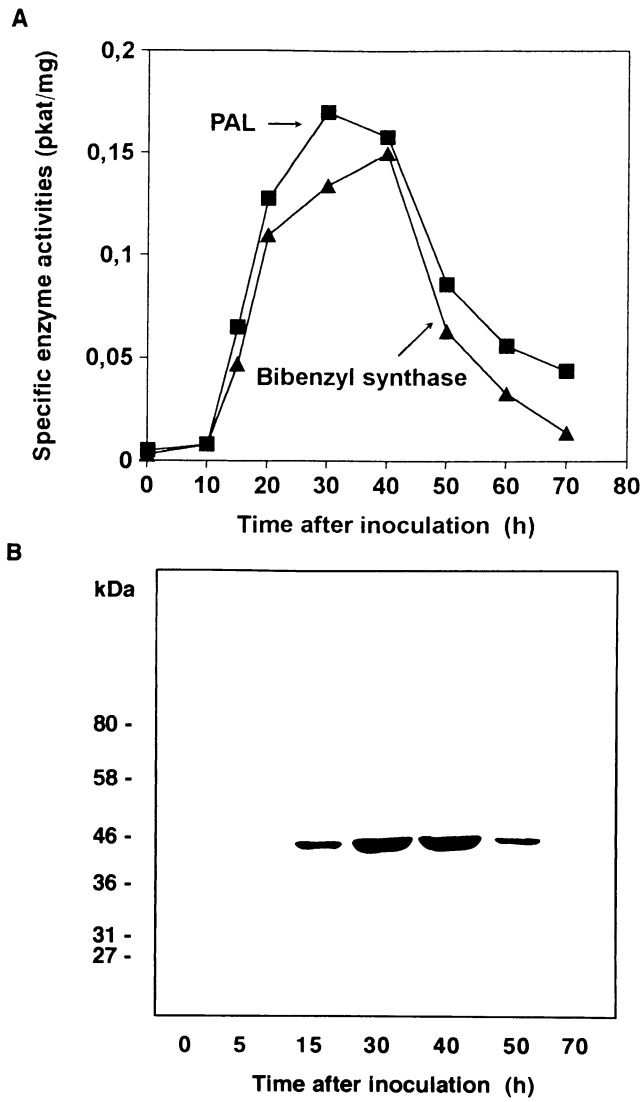

Fig. 3. Profile of bibenzyl synthase and phenylalanine ammonia-lyase (PAL) in leaves during the period subsequent to induction by treatment with *Botrytis cinerea*. **A,** Enzyme activity of bibenzyl synthase, in comparison to phenylalanine ammonia-lyase activity (100 relative units: 0.15 pkat × mg protein^{-1}). **B,** Immunodecoration using anti-bibenzyl synthase antiserum.

Infection of *Phalaenopsis* roots with *Rhizoctonia* species.

The incubation of roots of *Phalaenopsis* hybrid with *B. cinerea* did not result in enhanced levels of PAL or bibenzyl synthase in this tissue. However, *Rhizoctonia* sp. mycel caused an increase in bibenzyl synthase which could be demonstrated both by enzyme activities and immunodecoration of the protein (Fig. 4). Almost identical results were obtained with experiments using *R. crocorum* or *R. solani*, respectively (data not shown). To test whether the infection by *Rhizoctonia* species was tissue specific, *R. crocorum* was used to infect leaves of *Phalaenopsis* hybrid. The response to *R. crocorum* was much lower than the one observed with *B. cinerea* (Fig. 3).

DISCUSSION

We found that several enzymes involved in the dihydrophenanthrene pathway are inducible in orchids. In bulbils of *Dioscoraea macruora*, it was shown earlier that the formation of hircinol, a dihydrophenanthrene, was markedly increased by wounding and infection while the pathway to stilbenes, also present in this plant, was not susceptible to activation (Fritzemeier *et al.* 1984). Thus, dihydrophenanthrenes rather than stilbenes or phenanthrenes are subject to induction and may act as phytoalexins in these cases.

We have shown that at least three enzymes leading to the formation of the phytoalexin hircinol (see Fig. 1) are controlled by their environment. Furthermore, from the experiments it may be deduced that most orchids are capable of reacting in this way. *O*-Methyltransferases are known to be part of the process of forming more hydrophobic phytoalexins. As a rather general role, *O*-methylation is a late state of derivatization of a formerly built carbon skeleton. In the cases of stilbenes, we don't know any instance where the introduction of a second hydroxyl group at ring B or the *O*-methylation takes place at the level of phenylpropanoid precursors. Unlike the situation with those complex phenylpropane derivatives, the phenylpropanoid pathway to lignin does include *O*-methylation at the level of hydroxyated cinnamic acids. As exemplified by the hypersensitive reaction of tobacco to tobacco mosaic virus (Jaeck *et al.* 1992), *O*-methyltransferases may be subjects of induction. In line with this concept, the reaction sequence leading to the bibenzyls is characterized by an *O*-methylation at a rather late step in the biosynthetic pathway. The results (Table 1) prove that the *O*-methylation takes place prior to the formation of the tricyclic carbon skeleton (Fig. 1, via b and d).

The concomitant increase of the activities, and probably also of the respective proteins, of several enzymes involved in the dihydrophenanthrene pathway points to a signal chain addressing similar promoters on the genes encoding at least the following enzymes: PAL, bibenzyl synthase, and *O*-methyltransferase. As the turnover of *S*-adenosylmethionine requires the recovery of *S*-adenosylhomocysteine, we assume that *S*-adenosylhomocysteine hydrolase is equally induced. This was indeed indicated by the findings of a large number of *S*-adenosylhomocysteine hydrolase cDNA clones in a cDNA library derived from induced *Phalaenopsis* leaves (unpublished results). However, as to the pathway hypothesized *in toto*, we so far have no data on a cinnamate hydroxylase, a reductase and an acyl-CoA ligase, catalyzing the steps ahead of the bibenzyl synthase reaction (Fig. 1).

It has been argued (Harley and Smith 1983) that some mycorrhizal fungi, e.g., *R. solani*, are able to destroy orchinol and hircinol and that in symbiosis there may be a balance between phytoalexin formation by the plant and its inactivation by the fungus. A decisive point in our concept is that the response of young sterile plants is much higher than the reaction of older plants that were exposed to the environment of a normal greenhouse but otherwise grown under comparable conditions. At an early stage of colonization, the extent of bibenzyl synthase formation was much more pronounced compared to the response of an older plant. Mycorrhizal roots showed an extremely low level of bibenzyl synthase, indicating that the susceptibility of the plant to responding against the fungus is down-regulated. These mycorrhizal roots, however, still possessed the capacity to react,

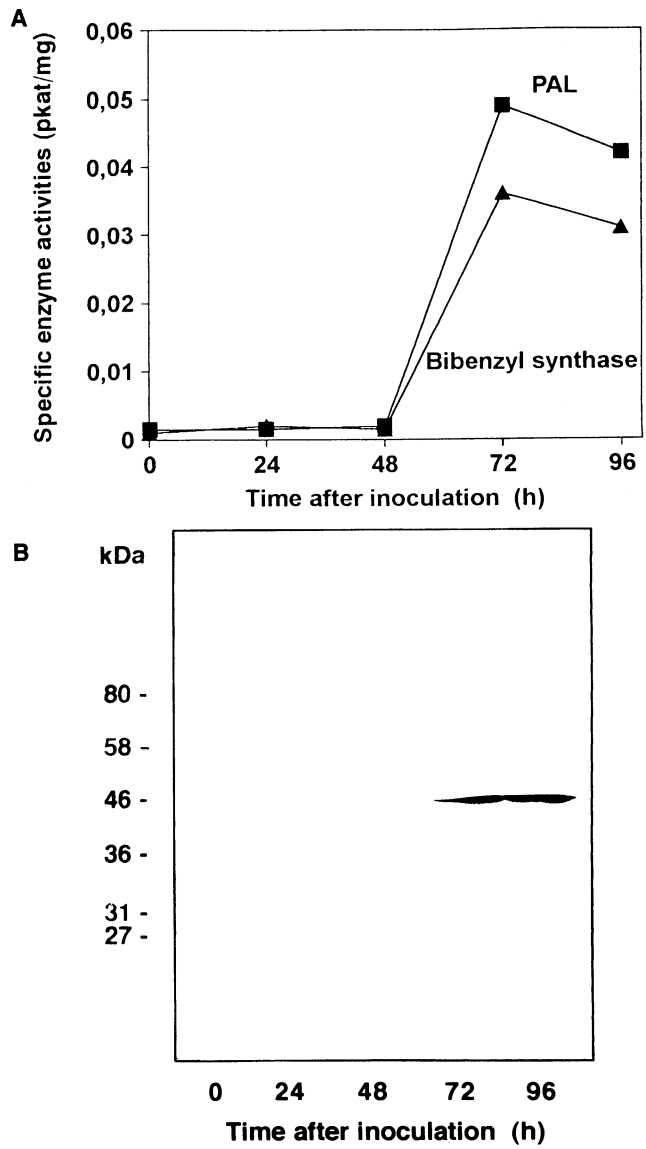

Fig. 4. Profiles of enzyme activities **(A)** and immunodetectable bibenzyl synthase protein **(B)**. Bibenzyl synthase and PAL in roots of *Phalaenopsis* hybrid infected with *Rhizoctonia* sp. One hundred relative units represent 0.05 pkat × mg protein^{-1}. For Western blots, each lane was loaded with 70 µg of protein.

e.g., when the mycorrhiza was destructed (Gehlert and Kindl 1991).

The functional role of phytoalexins during the stage of development of mycorrhiza is unclear. We hypothesized that phytoalexin production by the plant is reduced as the establishment of the mycorrhiza is progressing and the exchange of chemical signals between plant and fungus silences the responses of both partners.

MATERIALS AND METHODS

Chemicals.

Dihydro-*m*-coumaric acid and CoA-esters of cinnamic acids were synthesized according to (Fritzemeier and Kindl 1983). [2-^{14}C]-Malonyl-CoA (1.8 GBq/mmol), [*methyl*-^{14}C]*S*-adenosylmethionine (1.6 GBq/mmol), and [1-^{14}C]L-phenylalanine (1.8 GBq/mmol) were obtained from Amersham-Buchler (Braunschweig, Germany). Antiserum was raised against the bibenzyl synthase from *B. striata* (Reinecke and Kindl 1994). Goat anti-rabbit IgG conjugated with biotin and the conjugate of streptavidin and peroxidase were products of Amersham-Buchler. Goat anti-rabbit IgG conjugated with alkaline phosphatase was from Bio-Rad (Munich, Germany). Diaminobenzidine, NP-40, and Polyclar AT were products of Serva (Freiburg, Germany).

Fungal strains.

B. cinerea was obtained from E. Schloesser, Institute of Plant Pathology, Giessen, Germany, and was grown on potato-dextrose agar. *R. crocorum* (DSM 62993), *R. solani* (DSM 63010), and *Rhizoctonia* sp. (DSM 3706) were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Rhizoctonia* sp. was grown on oat flakes medium 189 (DSM catalogue of strains) while all other fungi were maintained on potato-dextrose agar 129 (DSM catalogue of strains).

Plant material, growth, and inoculation.

Rhizomes of the chinese earth orchid *B. striata* were obtained from Firma Küppers, Eschwege, Germany. Sterile plantlets of hybrids of *Phalaenopsis* and *Cymbidium* were from Orchideen Koch, Lennestadt, Germany. Both hybrids Schneestern (No. 1889) and Schöne Müllerin (No. 2446) derived from *Phalaenopsis amabilis*. The 4- to 24-wk-old plants were grown and maintained under sterile conditions on Phytamax orchid maintenance medium (Sigma, Deisenhofen, Germany) containing 1% (w/v) agar. Growth was under continuous white light (7,000 lx) at 27° C.

After the growth time indicated, the intact seedlings were either harvested or the leaves and roots were infected with a fungal suspension of *B. cinerea* conidia in 0.5% (w/v) sucrose. The suspension with *B. cinerea* contained 10^6 conidia per milliliter. Subsequent to infection, the plants were kept at 100% humidity and 27° C for the time indicated. Prior to the infection with mycelial suspension of *Rhizoctonia* species, the plants were removed from the agar using a platin loop and put on filter paper. Then, mycelial suspension was carefully applied to the roots.

Isolation of bibenzyls and other phenols.

Solvent systems were used for thin-layer chromatographic separations according to Fritzemeier and Kindl (1983). Conversion of dihydroxy-methoxy bibenzyl to hircinol was performed by administering 200 Bq of the *in vitro*-formed product (X) to slices of bulbs and incubation for 10 hr. Subsequently, the phenols were extracted from the slices and separated by thin-layer chromatography (TLC).

Enzyme assays.

Bibenzyl synthase activity was determined with 100 µM dihydro-*m*-coumaroyl-CoA and 20 µM [2-^{14}C]malonyl-CoA as substrates as previously described (Reinecke and Kindl 1994). The *O*-methyltransferase activity was assayed using 100 µM dihydro-*m*-coumaroyl-CoA, and 100 µM [*methyl*-^{14}C]S-adenosylmethionine as substrates. PAL activity was measured with [1-^{14}C]L-phenylalanine according to (Kindl 1970).

Protein extraction.

One gram of plant material powdered using liquid N_2 and 0.15 g of Polyclar AT were suspended in 1.0 ml of 20 mM Hepes-NaOH, pH 7.9, containing 2 mM mercaptoethanol. Homogenization was with an Ultraturrax three times 15 sec at full speed. The homogenate was centrifuged at 20,000 × *g* for 20 min at 4° C. The supernatant (0.8 ml) was passed through PD-10 (Pharmacia, Feiburg, Germany) equilibrated with the homogenization buffer. The protein peak contained in the first 1.2 ml after the void volume was collected, and 40 µl of this solution was used for the enzyme assay.

Western blot and other methods.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the bibenzyl synthase-containing protein extracts. Proteins were transferred to nitrocellulose by semi-dry blotting according to (Kyhse-Anderson 1984). Immunodecoration was performed using the biotin-streptavidin system. Briefly, following blocking with 5% (w/v) bovine serum albumin (BSA) in 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl (P1) for 1 hr, the first antibody was applied in P1 supplemented with 5% (w/v) BSA for 1 hr. Subsequent washings were with P1, 0.05% (w/v) NP-40 (twice, 15 min), and P1. Then, the second antibody, i.e., goat anti-rabbit IgG conjugated with biotin, was added in the presence of 5% (w/v) BSA for 1 hr. Washings were as before. Conjugate (0.15%) between streptavidine and peroxidase dissolved in P1 containing 5% (w/v) BSA was incubated for 1 hr with the blot. Following further washings, the enzyme reaction was started with 0.8 mg/ml diaminobenzidine and 0.03% (v/v) H_2O_2 in P1. After development of intensive color, the reaction was stopped with 0.1% (w/v) sodium azide. Alternatively, the visualization of bibenzyl synthase was with antibodies followed by incubation with goat anti-rabbit IgG conjugated with alkaline phosphatase as described by Blake *et al.* (1984).

Protein content was determined (Bradford 1976) using bovine serum albumin as the protein standard. Radioactivity was determined by liquid scintillation counting. Absolute radioactivity was calculated by a calibration program based on the channels ratio method.

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