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Communication

Rooting Cofactor Activity of Plant Phytoalexins[1]

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ABSTRACT

The pterocarpinoid phytoalexins, glyceollin, pisatin, and phaseollin, stimulated adventitious root formation in a mung bean rooting bioassay only in the presence of indoleacetic acid (rooting cofactor activity). Relatively low (5 to 50 micrograms per milliliter) concentrations of the phytoalexins were effective. The phytoalexins also increased the numbers of root primordia formed, therefore suggesting that they interacted with an early process in root formation.

Phytoalexins are inducibly formed higher plant metabolites that are antibiotic to certain potential plant pathogens. At least 100 plant species representing 21 families have been shown to accumulate phytoalexins in response to microbial infection and these comprise a defense mechanism analogous in some way to the immune response in animals (5, 10). The question arises, however, whether plants have developed the potential to produce phytoalexins solely for pathogen defense or whether they also have additional physiological functions in uninfected plants.

Yoshikawa *et al.* (9, 12) found that biosynthesis *per se* of the soybean phytoalexin glyceollin was induced in response not only to fungal infection but also to mechanical wounding, although its net accumulation in the latter case was quite low due to endogenous metabolizing enzymes. This led us to explore the possibility that phytoalexins may function in certain physiological processes at concentrations below their antibiotic activity, especially in those related to wound responses in plants. We report here that low concentrations of glyceollin and certain other phytoalexins react synergistically with IAA in a mung bean rooting bioassy to promote adventitious root formation (rooting cofactor activity, see Ref. 4), a wound response following hypocotyl cutting.

MATERIALS AND METHODS

Phytoalexins. Glyceollin was isolated and purified from fungus-challenged germinating soybean (*Glycine max* L. Merr.) seeds as described previously (11) except that Sephadex LH-20 column chromatography was included in the purification steps. The partially purified ethyl acetate fraction was evaporated to dryness, dissolved in 80% ethanol, and then applied to a column (2.5 × 60 cm) of Sephadex LH-20 (Pharmacia). The column was equilibrated and eluted with 80% ethanol. The eluted glyceollin fraction was further purified by preparative TLC as described previously (11). Glyceollin thus purified was composed of a mixture of glyceollin isomers and this mixture was used for the present experiment without further separation.

Purified phaseollin and pisatin were kindly donated by H. Oku, Okayama University.

Bioassay for Rooting Cofactor Activity. Seeds of mung bean (*Phaseolus aureus* Roxb.) were surface-sterilized in 1% NaOCl for 5 min and rinsed with running tap water. The seeds were heated at 60°C for 3 h to promote germination and then sown in moist vermiculite. The plants were grown in a growth chamber at 25°C under fluorescent lighting (about 2000 lux).

Ten-d-old mung bean seedlings were cut at hypocotyls, 3 cm below the cotyledonary nodes and freed of the remaining cotyledons. The cutting were placed in 20 ml of deionized H_2O in test tubes containing either one of the phytoalexins at the indicated concentrations with or without IAA (10 μg/ml). The plants were kept in the growth chamber with daily addition of deionized H_2O to maintain the original level (3 cm depth) of the solution in the tubes. Ten plants were used for each assay and the numbers of adventitious roots formed were counted at 7 d after incubation.

For counting the numbers of root primordia formed, hypocotyl segments (3 cm long) below the cotyledonary nodes were cut at 4 d after incubation. They were then placed in ethanol (50%):formalin:glacial acetic acid (100:6.5:2.5) and kept in the solution for 2 d to clear the hypocotyls segments. Root primordia in the cleared hypocotyl segments were visually detected as tiny dark spots. Identity of the spots to root primordia was confirmed by microscopic observation after making thin sections of the hypocotyl segments and staining them with hematoxylin.

RESULTS AND DISCUSSION

Typical adventitious root formation of mung bean seedlings in response to exogenous IAA and glyceollin is shown in Figure 1. In water, 4 roots were formed at the base of the cut hypocotyls (Fig. 1A). Glyceollin alone did not affect root formation (Fig. 1B), but acted synergistically with IAA, leading to more root formation (Fig. 1D) than the auxin alone (Fig. 1C). Our preliminary experiments revealed that the numbers of adventitious roots increased as IAA concentrations were raised from 5 to 50 μg/ml, but the most consistent and clearest effects of glyceollin were obtained at 10 or 20 μg/ml of IAA. The stimulative effect of glyceollin was observed at 5 μg/ml and 10 or 20 μg/ml produced the maximum (1.4–1.8-fold) stimulation of IAA-treated root formation (Table I). High concentrations of glyceollin (*e.g.* 100 μg/ml) were apparently phytotoxic since the plants wilted. Similar stimulation of the root formation in the presence of IAA was also observed with phaseollin and pisatin, phytoalexins produced by beans (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.), respectively (Table I).

Possible stages during adventitious root formation at which the phytoalexins interact were evaluated using glyceollin (Table

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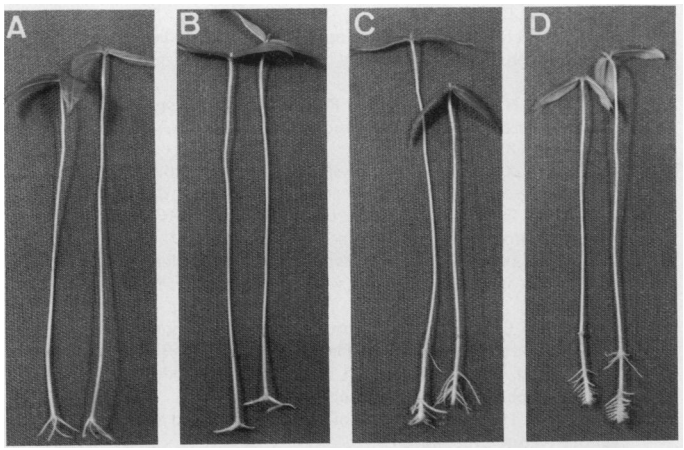

FIG. 1. Adventitious root formation in the mung bean rooting bioassay in response to exogenous indoleacetic acid (IAA) and the soybean phytoalexin glyceollin. A, Water only; B, glyceollin (20 μg/ml) only; C, IAA (10 μg/ml) only; D, glyceollin (20 μg/ml) plus IAA (10 μg/ml).

Table I. *Effects of Various Phytoalexins on Adventitious Root Formation in the Mung Bean Rooting Bioassay in the Presence or Absence of IAA*

Phytoalexin	Concentration of Phytoalexins	No. of Adventitious Roots[a]	
		Without IAA	With IAA (10 μg/ml)
	μg/ml	% of control	
Control (H_2O)		100 (4.1 ± 0.3)[b]	100 (8.3 ± 2.8)[b]
Glyceollin	1	101 ± 8	105 ± 7
	5	95 ± 2	141 ± 31
	10	104 ± 6	167 ± 17
	20	105 ± 12	177 ± 20
	50	113 ± 21	140 ± 34
	100	PT[c]	PT[c]
Phaseollin	5	100	160 ± 20
	10	97	194 ± 47
	20	113	192 ± 38
	50	100	165 ± 30
Pisatin	5	101	136 ± 28
	10	89	171 ± 25
	20	103	163 ± 31
	50	97	128 ± 22

[a] The numbers of adventitious roots formed were counted at 7 d after incubation. Means and standard errors are indicated where 3 to 7 replicate exeriments were run, and other values are means of two replicate experiments. [b] Actual numbers of adventitious roots formed per plant. [c] PT = plant toxicity; plant wilted in these solutions, possibly due to toxicity of the phytoalexin.

II). Under the experimental conditions, root primordia became visible microscopically and visually at 2 and 4 d, respectively, and then roots elongated to about 10 mm after 7 d incubation. Glyceollin in the presence of IAA promoted root formation when supplied at or before 2 d after incubation, but the effect was diminished when it was added at 3 d or later. Further, a nearly maximum effect of glyceollin was observed when it was present during the initial 2 or 3 d incubation. These results indicated that glyceollin affects the relatively early stages of root primordium formation but does not affect root elongation. Indeed, glyceollin and the other phytoalexins increased the number of root primordia in the presence of IAA (Table III) to the extents similar to the increases in the final numbers of elongated roots formed (Table I).

Table II. *Effect of Glyceollin That Was Present for the Limited Time Periods of Incubation on Adventitious Root Formation in the Mung Bean Rooting Bioassay in the Presence of IAA*

Time for Addition or Omission of Glyceollin after Incubation	No. of Adventitious Roots[a]
d	*% of IAA-control*
Addition of glyceollin[b]	
0	166 ± 36
1	171 ± 42
2	153 ± 25
3	107 ± 21
4	89 ± 16
Omission of glyceollin[c]	
1	122 ± 23
2	157 ± 18
3	168 ± 31

[a] The numbers of adventitious roots formed were counted at 7 d after initial incubation. Data are means of 3 replicate experiments and expressed as percent of each corresponding IAA-control described below. [b] The cut mung bean seedlings were initially placed in the solution containing IAA only and then transferred into the solution containing both IAA and glyceollin, or IAA only (IAA-control) at the indicated days of incubation. [c] The cut seedlings were initially placed in the solution containing both IAA and glyceollin, or IAA only (IAA-control) and then transferred into the solution containing IAA only at the indicated days of incubation. In all cases, IAA and glyceollin were added at 10 and 20 μg/ml, respectively.

Table III. *Effects of Various Phytoalexins on Root Primordium Formation in the Mung Bean Rooting Bioassay in the Presence or Absence of IAA*

Phytoalexin[a]	No. of Root Primordia[b]	
	Without IAA	With IAA (10 μg/ml)
	% of control	
Control (H_2O)	100 (4.0 ± 0.7)[c]	100 (9.1 ± 3.2)[c]
Glyceollin	105 ± 16	169 ± 7
Phaseollin	100 ± 5	173 ± 25
Pisatin	105 ± 19	155 ± 19

[a] Each phytoalexin was tested at 20 μg/ml. [b] The numbers of root primordia formed were counted at 4 d after incubation. Data are means of 3 replicate experiments. [c] Actual numbers of root primordia formed per plant.

The results presented here demonstrate non-antibiotic physiological activity for three phytoalexins. Although further study is necessary to fully elucidate the *in vivo* function of phytoalexins, the fact that both adventitious root formation and the synthesis of phytoalexins are induced after mechanical wounding supports the possibility that phytoalexins may indeed be involved in adventitious root formation. Furthermore, the induction of phytoalexin synthesis after wounding occurs within 1 d (1, 9, 12), consistent with their presumed role in early stages of root formation.

Despite induced phytoalexin synthesis, the net accumulation of phytoalexins in wounded plant tissues is generally quite low or undetectable, apparently due to the presence of phytoalexin degrading activity in the wounded tissues (3, 9, 12). It is likely, however, that phytoalexins accumulate to low concentrations in the wounded tissues, but are not detected due to low sensitivity of the analytical methods. These considerations lead to the idea that phytoalexins at low concentrations may be associated with

certain wound responses, but following pathogen infection they accumulate to high levels, due to the further activation of synthesis and blockage of degradation (9, 12).

Rooting cofactors are considered as endogenous substances capable of acting synergistically with IAA in the rooting of cut mung bean seedlings (4). Substances with cofactor activity have been isolated from several plant species and are usually phenolic compounds. Pterocarpan phytoalexins such as glyceollin, phaseollin, and pisatin are synthesized through phenolic compounds (6, 13). Since the cofactor activity of phytoalexins was about 10 times higher on a molar concentration basis than with simpler phenolic compounds (2), the induced synthesis of phytoalexins after wounding may constitute the conversion of phenolic compounds to more active ones.

Although the mechanisms by which phytoalexins act as cofactors have not been evaluated, considerable data (7, 8) as well as our own unpublished observations have indicated that antibiotic activity of the phytoalexins used in the present study arises primarily from interference with membrane systems of plant and fungal cells. Further study is necessary to elucidate whether their rooting cofactor activity is manifested through interaction with plant membranes or through other unknown mechanisms.

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