

## **Abscisic Acid is a Potent Inhibitor of Growth and Sporidial Formation in *Neovossia indica* Cultures: Dual Mode of Action *via* Loss of Polyamines and Cellular Turgidity**

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The inhibition of polyamine biosynthesis in *Neovossia indica* (Mitra) Mundkur by D,L- $\alpha$ -difluoromethylornithine (DFMO) caused an effective reduction of mycelial growth and sporidial production under *in vitro* conditions, which was reversed by ornithine application. Abscisic acid proved to be similarly effective, not only in inhibiting mycelial growth and sporidial formation but also the germination of teliospores, which constitute the primary inoculum of the pathogen. ABA-mediated inhibition resulted in decreased polyamine levels and loss of cellular turgidity of mycelial cultures. Scanning electron microscopy of ABA-treated cultures revealed extremely shrunken hyphae, in marked contrast to the turgid controls. It is suggested that the manipulation of ABA levels and/or tissue sensitivity in wheat could be a strategy to combat 'Karnal' bunt, the disease caused by *N. indica*.

**KEY WORDS:** Abscisic acid; polyamines; *Neovossia indica*; Karnal bunt; morphogenesis; disease resistance.

### INTRODUCTION

There is increasing evidence that both the pathogen and the host have the capacity to synthesize various growth regulators, and alterations in their levels and/or sensitivity as a result of plant-pathogen interaction are related to disease susceptibility or resistance reaction (3,6,9,10). The research work on these subjects has provided new insights into our understanding of pathogenesis and of the manipulation of disease resistance.

'Karnal' bunt, a serious disease of wheat caused by the fungus *Neovossia indica* (Mitra) Mundkur, has proved difficult to control, being seed-, soil- and airborne in nature (2). Wheat plants infected with the bunt fungus undergo major morpho-physiological alterations, mediated possibly by hormonal interactions between the pathogen and the host (10). A marked change in endogenous cytokinin levels has been observed (17). Healthy wheat plants treated exogenously with zeatin riboside or isopentenyl adenosine developed dwarf bunt-like symptoms (17). The involvement of other hormones such as auxins (8) and gibberellins (19) is less well understood. Although abscisic acid (ABA) has been implicated in plant responses to many fungal infections (6), its role in the development of infection by bunt fungi is not known. The fungicide triadimefon, which also has plant growth-regulating properties, appears to operate *via* its effects on ABA concentration in treated tissues (1,4). Interesting work on the involvement of polyamines, especially in regard to the possibility that control of fungal plant diseases might be achieved through inhibition of fungal polyamine biosynthesis, has been published (5,11).

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We reported earlier that it might be possible to control Karnal bunt disease of wheat through inhibition of polyamine biosynthesis by D,L- $\alpha$ -difluoromethylornithine (DFMO) (15). Whether a naturally occurring inhibitor in the host plant could inhibit polyamine metabolism of the invading fungus is not known. In the present study, the involvement of ABA in mediating such a response was investigated using *N. indica* cultures, which provide an amenable system to manipulate ABA levels and to disentangle the effects of hormonal changes induced by the host-pathogen interaction.

## MATERIALS AND METHODS

Karnal bunt-infected grains of wheat (*Triticum aestivum* L. cv. HD 2329) were sterilized with 1% sodium hypochlorite and then washed with distilled water. Teliospores of *N. indica* were removed by rupturing the pericarp of infected grains and sprinkled on sterile distilled water in petri dishes incubated at  $20 \pm 1^\circ\text{C}$ . After 10 days of incubation, the germinating teliospores were streaked on potato-dextrose-yeast extract (PDYE) agar slants to raise pure cultures of *N. indica* (14).

The filter-sterilized solutions of ABA, DFMO and ornithine were added to sterile PDYE broth to obtain required concentrations for studying their effect on growth and morphogenesis in *N. indica*. Such amended broth media were dispensed aseptically into 100 ml Erlenmeyer flasks (25 ml of medium in each). The control treatment consisted of culture medium (PDYE broth) without any amendment. The medium was inoculated with 2-mm-diam inoculum bits from actively sporulating cultures and incubated at  $20^\circ\text{C}$  for 15 days. There were six replications per treatment and all experiments were repeated twice.

The observations on number of sporidia/ml were taken with a hemocytometer. Dry weight of the fungal mycelium was recorded after drying at  $60^\circ\text{C}$  to constant weight. The effect of different ABA concentrations on teliospore germination was studied by floating teliospores on ABA-amended sterile distilled water and incubating at  $20 \pm 1^\circ\text{C}$  as described above. The germination counts were taken after 10 days of incubation.

### *Scanning electron microscope (SEM) studies*

For SEM studies, control and ABA (10  $\mu\text{M}$ )-treated mycelial culture (15 days old) of *N. indica* were fixed in 2% glutaraldehyde (0.05 M cacodylate buffer, pH 7.2, 24 h,  $4^\circ\text{C}$ ) followed by dehydration in an ethanol series and critical point drying. The mycelia were mounted on an aluminum stub with carbon tape and coated with gold. Specimens were critically examined with a Jeol J.S.M.-6100 SEM at 15 kv. Photomicrographs were taken on Indu (120  $\times$  35 mm) film.

### *Determination of polyamines*

Extraction and determination of polyamines from 15-day-old mycelial cultures were performed according to Szczotka (16). Mycelial cultures were extracted in 5% (v/v) cold  $\text{HClO}_4$  at a ratio of approximately 100 mg/ml  $\text{HClO}_4$  and centrifuged for 15 min at 9,000 g. The sediments were suspended, centrifuged and the supernatants pooled and transferred to Dowex-50 WX-4 (200–400 mesh) columns (90  $\times$  4 mm). The columns were washed with 40 ml of 0.1 M sodium-phosphate buffer (pH 8.0, in 0.7 M HCl) followed by 10 ml of 1N HCl. The polyamines were eluted with increasing concentrations of 5 ml HCl: putrescine 2 M HCl, spermidine 3 M HCl and spermine 4 M HCl. After evaporation of eluates to dryness, each sample was dissolved in 0.5 ml of water and 100- $\mu\text{l}$  portions

were placed on filter paper, dried, and sprayed with ninhydrin reagent comprising 1.0 g ninhydrin, 0.1 g cadmium acetate, 10 ml water, 5 ml acetic acid and 85 ml acetone. The spots became red after heating at 70°C for 60 min. Spots were extracted with 3 ml of solvent consisting of 0.2 g cadmium acetate, 10 ml water, 40 ml ethanol, and 50 ml acetic acid. Extraction was done at room temperature for 30 min, and absorbance was measured at 505 nm.

## RESULTS AND DISCUSSION

In the present study, we have shown that the addition of 1 mM DFMO to culture medium resulted in a tenfold reduction of sporidial formation which was restored by co-application of ornithine (10 mM) (Table 1), implying an essential role of polyamines in morphogenesis of *N. indica*, as reported previously (15). In fungi, the initial step in polyamine biosynthesis is the decarboxylation of ornithine to form putrescine, catalyzed by the enzyme ornithine decarboxylase (20). Apparently, ornithine is also acting as the precursor to polyamine biosynthesis in *N. indica* cultures, as its supplementation in the medium could reverse the effect of DFMO (Table 1). However, it is probable that the growth enhancement effect may be ascribed in part to the extra nitrogen availability. It is likely that a naturally occurring growth regulator could inhibit polyamine metabolism in a similar manner to DFMO. Alterations in the levels of plant growth substances in dwarf bunt-infected wheat plants have been reported (10,17,18). To investigate such a possibility, the inhibitory activity of potential bioregulators could be studied *in vitro* in fungal cultures.

TABLE 1. Effects of D,L- $\alpha$ -difluoromethylornithine (DFMO) and ornithine, alone and in combination, on growth and sporidial formation of *Neovossia indica* cultures

Treatment	Mycelial dry weight (mg)	Number of sporidia/ml of growth medium
1 mM DFMO	67a	$0.2 \times 10^5$ a
10 mM ornithine	114c	$4.3 \times 10^5$ c
1 mM DFMO + 10 mM ornithine	97b	$2.1 \times 10^5$ b
Control	100b	$2.6 \times 10^5$ b

Within columns, means followed by the same letter do not differ significantly at  $P=0.05$ .

TABLE 2. Effects of ABA on growth and development of *Neovossia indica* cultures

ABA treatment	Mycelial dry weight (mg)	Number of sporidia/ml of growth medium	Teliospore germination (%)
10 $\mu$ M	75b	$2.5 \times 10^4$ b	0.52a
50 $\mu$ M	64a	$1.0 \times 10^4$ a	0.47a
100 $\mu$ M	0	0	0
None (control)	234c	$2.75 \times 10^5$ c	16.20b

Within columns, means followed by the same letter do not differ significantly at  $P=0.05$ .

Fig. 1. SEM of 15-day-old mycelial cultures of *Neovossia indica* (A, 10  $\mu$ M ABA; B, untreated).

The present study revealed an important role for ABA as a potent inhibitor of growth, sporidiogenesis and teliospore germination of *N. indica* under culture conditions (Table 2). A few germinated teliospores which could be observed in ABA-amended medium did not bear any sporidia at all, *i.e.*, the promycelium remained abortive (visual observations). ABA at 100  $\mu\text{M}$  and above completely suppressed the pathogen growth *in vitro* (Table 2). It should be remembered that ABA has been implicated in the resistance to infection by many viral, bacterial and fungal pathogens (6).

In view of the important role of polyamines in growth and development of *N. indica* (15), the effect of ABA on polyamine levels of 15-day-old cultures was studied. It is worth noting that 10  $\mu\text{M}$  ABA application resulted in markedly reduced endogenous levels of different polyamines, *i.e.*, putrescine, spermidine and spermine (Table 3). The amounts of spermidine and spermine were comparatively higher and showed a greater per cent decline than putrescine. To the best of our knowledge, such an effect of ABA on fungal polyamine biosynthesis has not been shown previously. Further work on reversal of ABA's effect by exogenous polyamines applied singly or in combination is being pursued in our laboratory.

TABLE 3. Effects of 10  $\mu\text{M}$  ABA treatment on polyamine levels of *Neovossia indica* cultures

ABA treatment	Polyamine contents ( $\mu\text{g/g}$ dry weight)		
	Putrescine	Spermidine	Spermine
10 $\mu\text{M}$	181.5 $\pm$ 7.8	458.2 $\pm$ 12.3	538.7 $\pm$ 13.4
None (control)	298.9 $\pm$ 9.9	1015.4 $\pm$ 18.4	1041.9 $\pm$ 18.6

An additional factor which may be involved in growth inhibition is the loss of cellular turgidity by mycelial hyphae of *N. indica* cultured in the presence of 10  $\mu\text{M}$  ABA. The water content of ABA-treated cultures fell to 13% from the control value of 70% in untreated cultures. This was corroborated by SEM observations wherein the ABA-treated cultures showed extremely shrunken and squeezed hyphae, in marked contrast to the turgid hyphae of the untreated control (Fig. 1). Evidence has been presented that exogenous ABA controls seed germination by limiting water uptake to embryos (12) and possibly affects cell wall extensibility (13) or membrane rigidity (7) by a biophysical interaction with phospholipids.

Collectively, our results indicate that ABA acts as a potent inhibitor of growth and development in *N. indica* by affecting water relations and polyamine metabolism. Genetic variation in wheat ABA levels and/or responsiveness may be one of the possible strategies to combat this pathogen.

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