EFFECT OF BENZYL AMINO PURINE ON THE PATHOGEN GROWTH AND DISEASE DEVELOPMENT OF TARO LEAF BLIGHT CAUSED BY *PHYTOPHTHORA COLOCASIAE*

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SUMMARY

Cytokinins are implicated in a wide variety of developmental and physiological processes in plants including host-pathogen interactions. The effect of cytokinin benzyl amino purine (BAP) on *Phytophthora colocasiae* mycelium growth was investigated *in vitro* and *in planta*. BAP inhibited the growth of *P. colocasiae* under *in vitro* conditions and a corresponding decrease in endo β-1,3 glucanase (EBG) activity was observed in a dose-dependent manner. The BAP mediated inhibitory effect of *P. colocasiae* mycelium growth was overcome by supplying the media with an external source of EBG. Results also showed that BAP has inhibitory effect on disease development in taro leading to a reduction in disease severity which correlated with increasing BAP concentrations. It is concluded that the use of BAP could be an important strategy in the control of taro leaf blight.

Key words: Benzyl amino purine, β-1,3 glucanase, cytokinins, taro, *Phytophthora colocasiae*, taro leaf blight.

INTRODUCTION

Taro (*Colocasia esculenta* (L.) Schott), a tropical aroid is an important staple or subsistence crop for millions of people in developing countries, especially in Africa and south east Asia. Leaf blight of taro, caused by *Phytophthora colocasiae* is the most destructive disease of *Colocasia*. It has become a limiting factor for taro production in all taro growing-countries causing yield loss of 25-30 % (Jackson *et al*., 1980; Thankappan, 1985; Misra and Chowdhury, 1997). Taro leaf blight symptoms appear as small, water soaked spots, which increase in size and number. Under cloudy weather conditions with intermittent rains and temperature around 28ºC, the disease quickly spreads across entire fields giving them a blighted appearance. Metalaxyl- and mancozeb-based fungicides have proved effective in controlling taro leaf blight but the waxy leaf surface and the occurrence of the disease during rainy season makes fungicidal sprays ineffective (Misra, 1999). Moreover, these treatments are costly, soil microorganisms rapidly degrade metalaxyl, contaminanting the surrounding water and soil, and development of resistance against the fungicides is a possibility. Thus, there is a need to develop integrated management strategies to combat this disease using natural and environmentally friendly mechanisms.

The role of plant growth regulators in pathogenesis has not been widely studied but there is some evidence that both the pathogen and host plant have the capacity to synthesize various growth regulators whose level varies as a result of plant-pathogen interactions (Jameson, 2000; Serezhkina *et al*., 2004). For example, fluctuations in the level of growth regulators are related to disease susceptibility or resistance in wheat cultivars infected with powdery mildew (Serezhkina *et al*., 2004). It was shown that some fungal pathogens are not only able to increase the level of indole-3-acetic acid in their respective hosts, but are themselves capable of producing this auxin which is directly released into soil (Agrios, 1997). Furfurylamine, 1, 2, 3, 4 tetra-*o*-acetyl-β-D-glucopyranse, adipic acid monoethyl ester (FGA), a mixture of three analogues of plant growth regulators has direct anti-microbial activity against filamentous fungi like *Alternaria solani* and *Botrytis cinerea*, and the oomycetes *P. capsici* and *P. citrophthora* (Flors *et al*., 2004). Naphthalene acetic acid reduces disease severity of *Sclerotinia sclerotiorum* (white mold) attacks to bean and cucumber plants (Al-Masari *et al*., 2002). It was also shown that exogenous cytokinins at high concentrations can effectively suppress the growth of the barley powdery mildew pathogen and induce abnormalities in the morphology of fungal growth tubes and appressoria (Mishina *et al*., 2002).

These findings clearly suggest that phytohormones play significant role in ontogeny of pathogens. Nevertheless, no evidence is available supporting a direct role of growth regulators in inhibiting mycelium growth and necrotic lesions development in taro plants during infection caused by *P. colocasiae*. Therefore, it seemed important to analyse the role of growth regulators in the context of taro leaf blight pathogen. The present study was aimed at investigating the role of BAP, a synthetic

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cytokinin in the interaction of *P. colocasiae* and its host plant, to explore its potential in controlling taro leaf blight disease.

MATERIALS AND METHODS

Chemicals. All chemicals were analytical grade and purchased from Sigma (India) and Merck (India).

Biological materials. Taro cv. Telia plants grown from tubers, were planted in pots containing soil and *Trichoderma* enriched compost. Taro leaves of the same age were used for all experiments. Taro leaf blight infected leaves were collected from plants with symptoms. Leaf tissue fragments 2-3 cm in size were excised from lesion margins, sterilized in 1% sodium hypochlorite for 2 min, rinsed twice with sterile distilled water and placed onto a *Phytophthora* selective medium, i.e. rye agar amended with 20 mg l^{-1} rifamycin, 200 mg l^{-1} vancomycin, 200 mg l^{-1} ampicillin, 68 mg l^{-1} pentachloronitrobenzene and 50 mg l^{-1} 50% benlate. Following incubation in Petri dishes for 4 to 5 days at 20ºC, mycelia grown from leaf fragments was transferred to and maintained on potato dextrose agar (PDA; 250 g l^{-1} potato, 20 g l^{-1} dextrose and 20 g l^{-1} agar). For spore generation, mycelia of *P. colocasiae* were grown on V8 agar medium (50 g l^{-1} V-8 juice, 0.2 g l^{-1} $CaCO_3$ and 20 g l^{-1} agar) at 24ºC for 4 days. Sporangia were induced by transferring two pieces of culture blocks (10×10×3 mm) in 10 ml sterile distilled water in Petri dishes for 2 days under white fluorescent light (Aragaki *et al*., 1967). Zoospores were released from sporangia by chilling at 5°C for 30 min. After filtration through muslin, zoospore concentration was adjusted to 10^5 spore ml^{-1} with a haemocytometer.

Pathogenicity assay. Specific virulence of the *P. colocasiae* isolate was determined by inoculation of 4 cm discs excised from the median part of 30-40-day-old taro leaves. Leaf discs were placed adaxial side down on media in Petri dishes containing 15 g l^{-1} agar and the abaxial surface was inoculated with a 20 µl aliquot of a suspension containing approximately 2×10^3 spores. Petri dishes were incubated for 7-10 days in a moist chamber at 28°C. These assay were done in triplicate and repeated twice.

Evaluation of mycelial development on growth regulator plates. Pieces (1 cm diameter) of mycelia from 6-day cultures of *P. colocasiae* were grown in Petri dishes of PDA agar supplemented with 250 mg l^{-1} filter-sterilized chloramphenicol and BAP at either 0, 50, 100, 200, 300, 400, 500 or 600 µM at 28°C. Colony diameter was recorded at two-day interval. In another set of experiments, PDA plates amended with 200 and 400 µM of BAP and with laminarinase (β-1,3 glucanase activity: 5 units mg^{-1} from *Penicillium* sp.) at 0, 0.05 and 0.1 units ml^{-1} concentration were used to analyse the effect of β-1,3 glucanase (EBG) in counteracting the BAP-mediated growth of *P. colocasiae*. Mycelium growth rate (cm^2/day) in the plates was calculated with the following equation (Melinda *et al*., 1991):

$$R= \{[(D/2)^2 - (d/2)^2] \times p\}/ T$$

Where R = mycelium growth rate; D = average diameter of the colony (cm); d = disc diameter (cm); p = 3.14, and T = incubation time (days).

Quantification of β-1, 3 glucanase activity of *P. colocasiae* and taro. Fifty mg of *P. colocasiae* mycelium grown on and collected from PDA plates amended with 200 and 400 µM concentration of BAP, were ground in liquid nitrogen with mortar and pestle and suspended in 50 mM Na acetate (pH 5.2). The extract was centrifuged at 5000 g for 15 min. Pellets were resuspended in the same buffer and the extraction was repeated twice. Samples were assayed for EBG activity using laminarin as substrate, according to Mutaftschiev *et al*. (1997). The amount of reducing sugars liberated as a result of EBG action on laminarin substrate was estimated according to Nelson's method (1944). The enzyme activity was expressed as the amount of glucose released per ml of enzyme extract per unit time. For quantification of taro's EBG, leaf discs (4 cm diameter) were cut from young taro leaves with a cork-borer and put in Petri dishes containing different concentrations of BAP solution (50, 100, 400 µM) or water as a control.

In another set of experiments, cell wall glucan elicitor from *P. colocasiae* was isolated as described by Ayers *et al*. (1976). Taro leaf discs (4 cm diameter) were treated with the glucan elicitor after gently pricking the abaxial surface and placed in Petri dishes containing different concentrations of BAP solutions (1, 10, 100 and 200 µM) or in water. β-1,3 glucanase enzyme extract was prepared from tissue at 12, 24, 48 and 72 h as described by Molano *et al*. (1977). Samples were assayed for BGA using laminarin as substrate as described above. The experimental design was completely randomized and consisted of three independent experiments. All tests for significance were conducted at the $p \leq 0.05$ level.

BAP treatment and disease development. Pot-grown taro plants were placed in an illuminated growth chamber (300 µE m^{-2} s^{-1}) with 12 h photoperiod at 28°C for 4 days, after which leaves of the same age were inoculated on their abaxial surfaces with a 50 µl suspension containing approximately 500 sporangia. Treated leaves were further incubated at 27°C with 85% humidity in the same light condition, to allow symptoms to develop. Diseased leaves were detached and the diameter of lesions recorded. Diseased leaves were placed in plastic boxes containing different concentrations of BAP (200, 400, 600 and 800 µM) or water (control) and incubated at

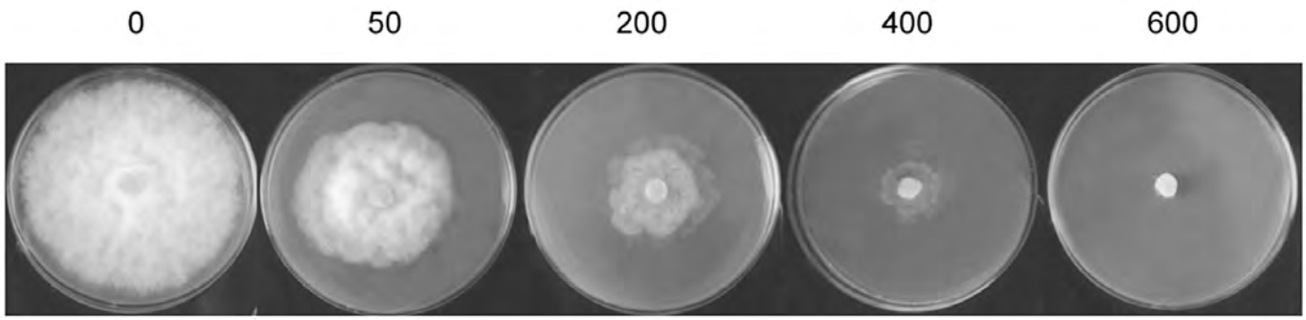

Fig. 1. *In vitro* inhibitory activity of BAP on *P. colocasiae* colony growth. The concentration of BAP is given in µM.

28°C with 12 h photoperiod. Evaluation of disease development on taro leaves was assessed at 2-day intervals by careful examination of the increase in lesion size. Disease growth rate was calculated as in the case of mycelial growth rate. The experimental design was randomized and consisted of three independent experiments. All tests for significance were conducted at the $p \leq 0.05$ level.

RESULTS

Pathogenicity assay. In a preliminary inoculation trial, the *P. colocasiae* isolate collected from infected taro leaves proved to be pathogenic to the susceptible taro cultivar used in this experiment.

Effect of BAP on the growth rate of *P. colocasiae* under *in vitro* condition. BAP decreased the growth rate of *P. colocasiae* mycelium grown on PDA at 50, 100, 200, 300, 400, 500 and 600 µM concentrations, compared to the control. Growth decreased with increasing BAP concentrations and, at 600 µM concentration of BAP, it was completely inhibited (Fig. 1).

The effect of BAP on disease severity in taro. The development of leaf blight disease in taro leaves treated with different concentrations of BAP was evaluated by measuring the growth rate (cm^2/day) of lesions 6 days after detachment of symptomatic leaves. The rate of disease development was calculated by subtracting the average diameter of lesions after 6 days from the size of lesions at the time of leaf detachment. BAP significantly reduced the development of leaf blight disease on detached leaves treated with 200, 400, 600 and 800 µM of BAP compared to control. This effect was dose-dependent for BAP concentrations of 200 to 800 µM (Table 1).

Effect of BAP on β-1,3 glucanase activity of taro. Results indicated that treatment of taro leaves with BAP, regardless of its concentration, does not modify EGB activity in taro (not shown). However, when taro leaves pre-treated with *P. colocasiae* cell wall glucan elicitor were further treated with BAP, a differential effect of BAP on EBG activity in taro was observed. Low concentration of BAP (1 and 10 µM) increased EBG activity in taro leaves pre-treated with *P. colocasiae* cell wall glucan, whereas higher concentrations of BAP (100 and 200 µM) decreased EBG activity. This decrease was more than two and three fold compared to lower concentrations of BAP (Fig. 2).

Table 1. Effect of BAP on leaf blight lesions growth rate on taro leaves.

Concentration of BAP (µM)	Rate of disease development (cm^2 / day)
0	4.1±1.0
200	2.0 ±0.8
400	0.8±0.2
600	0.3 ±0.1
800	0.1±0.05

* Result is shown as mean of four replicates ± standard deviation

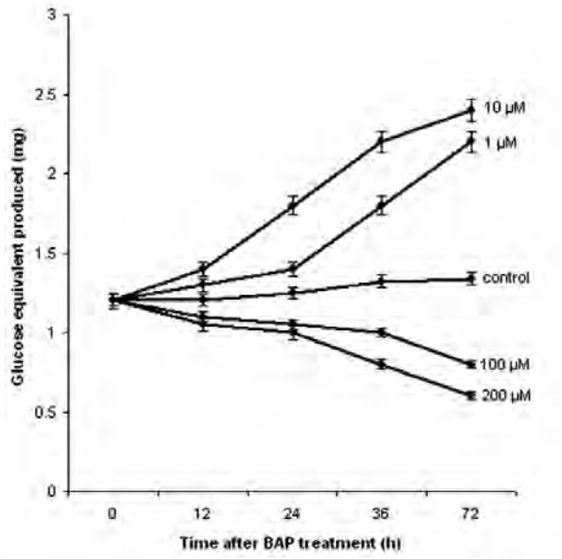

Fig. 2. Effect of BAP on β-1,3 glucanase activity of taro pretreated with cell wall glucan elicitor from *P. colocasiae* (Concentrations of BAP used in the study are indicated right).

Effect of BAP on β-1,3 glucanase activity in the mycelia of *P. colocasiae*. EBG activity of *P. colocasiae* grown on PDA plates amended with 200 and 400 µM of BAP was assayed. In the presence of 200 and 400 µM of BAP, the decrease in EBG activities were 66±2 and 80±3%, respectively, as compared to control plates (Fig. 3). Thus it was hypothesised that *P. colocasiae* grown on media amended with BAP, could be restored to its normal growth rate if the media are further supplemented with EBG. The growth rate of *P. colocasiae* on 200 and 400 µM BAP plates containing EBG, was found to increase when compared to the control (Fig. 4).

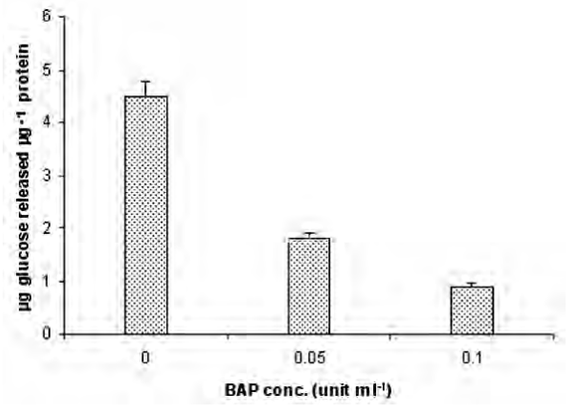

Fig. 3. Effect of BAP on β-1,3 glucanase activity in extracts from mycelia of *P. colocasiae*.

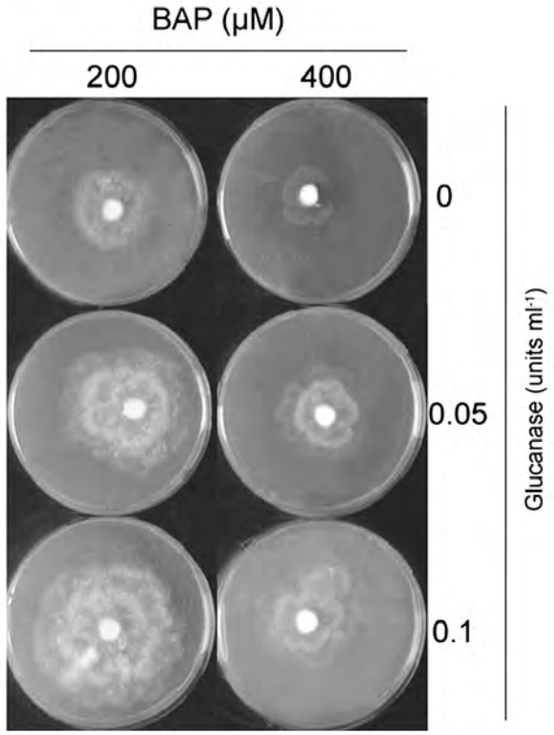

Fig. 4. Effect of laminarinase on *P. colocasiae* growth on PDA media amended with 200 or 400 µM BAP and containing 0.05 or 0.1 unit ml^{-1} laminarinase.

DISCUSSION

The present study has shown the important role of BAP as a potent growth inhibitor of *P. colocasiae* on taro plants and *in vitro*. BAP at 400 µM and above completely suppressed the pathogen growth *in vitro*. When taro leaves were treated with 200 to 800 µM of BAP, decrease in disease severity was observed. The extent of reduction in disease severity *in vivo* and growth of *P. colocasiae* under *in vitro* conditions were found to be dose dependent. This observation led us to investigate the possible mechanism that plays a major role in the growth retardation of *P. colocasiae* under *in vitro* and *in vivo* conditions. It is widely accepted that glucanase and chitinase form part of the active defence mechanism in plants (Van Loon and Van Strien, 1999). Their expression increases upon infection by pathogens and is positively correlated with growth inhibition of fungi *in vitro* and *in vivo* (Masoud *et al*., 1996). Chitinase is widespread in many species of higher plants and chitin is a major component of the cell wall of many fungi (Bartnicki-Garcia, 1968). On the other hand, cell wall of *Phytophthora* species chiefly consists of glucan (Tokunaga and Bartnicki-Garcia, 1973). Therefore, the role of chitinase in resistance to *P. colocasiae* was not investigated in this study.

Penetration and colonization of the host tissue by *Phytophthora* species is enhanced by secretion of enzyme capable of degrading of middle lamella of host tissue (McIntyre and Hankin, 1978). Optimum pH range for enzymes pectinase, pectinmethylesterase (Lumsden, 1976), cellulase, hemicellulase (Barkai-Golan, 1974; Lumsden, 1976), endopolygalacturonases (Gotesson *et al*., 2002) and proteolytic enzymes (Khare and Bompeix, 1976) is 4.3-5.5. The pH value of BAP solution used in this study was 5.0-5.2. Thus, there was no possibility of reduction of cell wall degrading enzymes. We observed the depletion of fungal EBG activity to the extent of 66±2 and 80±3% in extracts from *P. colocasiae* growing in media containing 200 and 400 µM BAP, respectively. Furthermore, the BAP-mediated inhibitory effect of *P. colocasiae* mycelium growth rate was overcome by supplementing the media with 0.05 and 0.1 units ml^{-1} EBG. Again we observed that restoring of growth rate by exogenous EBG supplements was dose dependent. Several reports have described the secretion of EBG by growing fungal mycelia and their role in turgor driven emergence of hyphal branches (Wessels, 1984) as well as the correlation between the amount of EBG secretions and cell wall strength in oomycete hyphae (Money and Hill, 1997). Based on this, we predicted that external EBG activity is able to restore the branching of apical hyphae and mycelial growth. Preliminary experiments showed that exogenously applied BAP could be effectively transported inside host tissue. Thus based on this observation we studied the effect of BAP in inducing host cell glucanase that antagonised the pathogen. Our re-

sults showed that treatment with BAP, irrespective of its concentration did not influence the glucanase activity of taro plant unless host arsenal mechanism was triggered by elicitor treatment. Our findings indicate that BAP acts as a potent inhibitor of growth of *P. colocasiae* by affecting its endogenous EBG activities necessary for promoting turgor driven emergence of hyphal branches and mycelial growth.

The BAP-mediated growth retardation of *P. colocasiae* provides evidence for its efficacy in the biological control for disease prevention and thus genetic variation of BAP levels in taro may provide a new strategy to combat taro leaf blight. The production of secondary metabolites such as cytokinins is widespread in the plant kingdom. These cytokinins promote leaf expansion and the transcription of genes of photosynthetic apparatus. This process leads to large leaves with high photosynthetic capacity in high light that maximizes carbon gain per unit of resources available for photosynthesis (Lambers *et al*., 1982). Its involvement in plant pathogenesis is not widely studied. However there are some reports supporting both inhibitory (Shinshi *et al*., 1987) and inducer effect of cytokinin on defence responses during plant-pathogen interactions (Goosens and Vendrig, 1982). It would be interesting to test the involvement of cytokinin in pathogen defence response using transgenic taro plants expressing an increased cytokinin level.

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