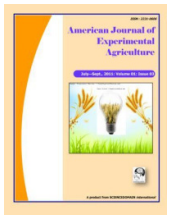

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Induction of Resistance in Cocoyam (*Xanthosoma sagittifolium*) to *Pythium myriotylum* by Corm Treatments with Benzothiadiazole and its Effect on Vegetative Growth

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Authors' contributions

This work was carried out in collaboration between all authors. Author DO designed the study, wrote the protocol and wrote the first draft of the manuscript. Author NDT reviewed the experimental design and all drafts of the manuscript. Authors DO and BEA conducted laboratory and greenhouse activities. Author BEA performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Root rot disease of cocoyam (*Xanthosoma sagittifolium*) caused by *Pythium myriotylum* (Oomycete) is a major constraint in cocoyam production in many countries in the world. Benzothiadiazole (BTH), a systemic acquired resistance inducer, was used in two concentrations (15 and 50 mg/L) as corm soaking treatment for 24 h duration to test for its effect on root rot disease resistance and vegetative growth of cocoyam under greenhouse conditions. The fungicide Metalm 72 WP (metalaxyl + cuprous oxide) was used as standard chemical control. Corm treatment with BTH significantly reduced cocoyam root rot disease (CRRD) severity compared with the pathogen-inoculated control. This was expressed by a disease index percentage of 37 and 20 for 15 and 50 mg/L BTH respectively after 28 days post-inoculation as opposed to the control with a disease index of 60. BTH at 50 mg/L was found to be more effective than the fungicide in inducing significant levels of resistance against *P. myriotylum*. In the absence of the pathogen, BTH and the fungicide applied as

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corm soaking treatment did not significantly influence the chlorophyll content. BTH significantly reduced shoot and corm/root dry weights compared to the control and fungicide respectively. However no visible phytotoxic effect was observed. BTH was tested for possible effects on *P. myriotylum in vitro*. There was no inhibition of mycelial growth with 15mg/L of BTH in the growth media in comparison with the control. No remarkable antifungal activity was observed with 50 mg/L. The promising effect of BTH against CRRD indicates that this compound may be future alternatives to traditional chemicals for disease control.

Keywords: Benzothiadiazole; induced resistance; corm treatment; Xanthosoma sagittifolium; Pythium myriotylum; vegetative growth.

1. INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium*), one of the most important edible tuber crops to millions of people in the tropics and subtropics, has been severely affected by a root rot disease caused by a soil-borne fungus, *Pythium myriotylum* [1,2]. Symptoms of cocoyam root rot disease (CRRD) include: slow rate of leaf production, premature yellowing of young leaves and initial water soaked lesions at the root tips that gradually spread to the entire root system resulting in root rot. Plants become stunted when attacked at the early growing stage. Yield losses in some infected cocoyam plantings in Cameroon were reported to be as high as 90% [1]. Acceptable cocoyam varieties resistant to CRRD are not available. Current control methods are based on cultural practices such as long interval of bush fallowing, roughing of diseased plants, crop rotation, etc and the use of fungicide which cannot consistently suppress the disease [3]. In the absence of cocoyam cultivars resistant to this disease and lack of efficient control measures, development of alternative or complementary approaches for management of this disease is highly desirable.

In this context, the induction of resistance using the host defence mechanisms is of practical application against plant diseases. This mode of resistance is increasingly attracting attention because of its potential usefulness in crop protection. The prospect of broad-spectrum disease control using the plant's own resistance mechanism has led to increasing interest in the development of agents which can mimic natural inducers of resistance [4]. Systemic acquired resistance (SAR) inducers have been shown to control many plant pathogens in agricultural production. BTH or acibenzolar-S-methyl (ASM) is among the most commonly used elicitors. When applied to the plant, it can induce accumulation of pathogenesis related-proteins (such as chitinases, glucanases, peroxidases) and lead to reduced incidence and severity of several diseases on many crops [5].

However, the scope of resistance activation by BTH is crop-specific. Optimal integration of BTH in disease control measures, therefore, needs to be carefully assessed for each crop. Very limited information is available about SAR inducers that may provide protection of cocoyam plants against CRRD. Previous studies indicated that BTH applied as foliar sprays was an efficient elicitor of some defence reactions in cocoyam and enhanced the activities of peroxidase, polyphenol oxydase as well as phenolic compounds [6]. BTH is usually applied as foliar sprays, but it can also induce systemic resistance when applied as seed imbibition in oilseed rape [7], cashew [8] and melon [9]. In cocoyam, the corms are the most common material used by farmers for cultivation. These corms are primary sources of *P. myriotylum* inocula. Consequently, inducer application by corm treatment suggests a suitable procedure for obtaining maximum protection against CRRD during seedling emergence and early growth stages. Therefore, the aim of this study was to test the effect of corm application of BTH on resistance to *P. myriotylum* in cocoyam and on plant vegetative growth. The direct effect of BTH on *in vitro* mycelial growth of *P. myriotylum* was also investigated.

2. MATERIALS AND METHODS

2.1 Experimental Location and Materials

The work was carried out in 2011-2012 in the greenhouse at the Jay P. Johnson Biotechnology Laboratory of the Institute of Agricultural Research for Development (IRAD), Ekona Regional Centre, in the South West Region of Cameroon.

Plant materials composed of corms of the highly susceptible cocoyam cultivar (*X. sagittifolium* cv.

White) were obtained from farmers' fields in Ekona (South West Region, Cameroon). Corms were trimmed with a knife to an average weight of 71g, disinfected in 5% (v/v) sodium hypochlorite (commercial bleach, 9% chlorine) for 30 minutes and thoroughly rinsed.

The chemical inducer and fungicide used in this study included:

Bion (WG 50) with 50% BTH was dissolved in distilled water. Metalm 72 WP (1 metalaxyl: 5 cuprous oxide, w/w) is a product distributed by AGROCHEM Douala (Cameroon) and was prepared by dissolving the required quantity in distilled water. It is a systemic and contact fungicide which is effective against *Pythium* spp. Concentrations of BTH used in this study are concentrations of the active ingredients of the product.

2.2 Effect of BTH as Corm Treatment on Severity of CRRD

Disinfected corms were soaked into different concentrations of BTH (15 and 50 mg/L) for 24 h. The concentrations were based on the results from preliminary trials in the laboratory. Corms soaked for 12 h in the suspension of 3.3 g/L of Metalm 72 WP were used as standard. Corms of healthy and disease control plants were soaked in distilled water for 24h.

Plant experiments were carried out in disease-conducive volcanic soil collected from Ekona. Soil was sterilized at 121℃ for 20 minutes in an autoclave and cooled for 2 days prior to use. Chemically treated and control corms were planted in polybags (30.5 x 21.2 cm) containing 3 kg of sterilized soil. Each polybag contained 1 corm. The experimental set-up was a completely randomized design (CRD) with 5 corms per treatment, including a healthy and a disease control. Plants were grown in greenhouse at 28ºC and watered when necessary to maintain moisture content of the soil substrate. The experiment was conducted three times.

Four-week-old plantlets, grown on sterilized soil, were used for challenge-inoculation with the pathogen. 5-7 day-old *Pythium* cultures were blended using a commercial blender in distilled water to produce a stock suspension of mycelia stands. The inoculums concentration was adjusted to about 1250 propagules per gram of soil. Inoculation consisted in spreading 100 ml of mycelia suspension on the soil of the potted plants.

Disease severity was determined the 28th day after inoculation on fully-opened leaves based on a rating scale from 0 to 4: 0 = 0%, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100% of foliage with yellowing [1] and disease index percentage (DI%) was estimated using the formula as follows:

$$DI\% = ((n1+2n2+3n3+4n4))/4N \times 100$$

Where:

n1 = number of plants with scale 1
n2 = number of plants with scale 2
n3 = number of plants with scale 3
n4 = number of plants with scale 4
N = total number of plants sampled

2.3 Effect of Corm Treatment with BTH on Plant Vegetative Growth

Disinfected corms were soaked into BTH or fungicide solution as described previously. For control plants, corms were soaked in distilled water for 24 h. The experimental set-up was a completely randomized design (CRD) with 10 corms per treatment, including a control. The experiment was repeated three times. Data was collected on the following parameters:

The daily numbers of sprouted corms were recorded until a constant count was achieved and the time taken for 50% sprouting of corms (E50) calculated according to the following formula [10]:

$$E50 = ti + (N/2 - ni)\ ((nj - ni))/((tj - ti))$$

Where:

N = final number of emerged seeds (sprouted corms)
ni and nj = cumulative number of seeds emerged by adjacent counts at times ti and tj
ti and tj = Initial time and final time, respectively

Chlorophyll content: Chlorophyll content in fresh leaves was estimated using the method described in [11]. For chlorophyll extraction, 1g fresh tissue from interveinal leaf area was cleaned several times with distilled water and homogenized in mortar and pestle with 90% (v/v) acetone. The extract was then centrifuged at 2500 rpm for 10 minutes. The optical density

(OD) of the supernatant was read and recorded at 663 nm and 645 nm (corresponding to chlorophylls a and b respectively) using a spectrophotometer.

Chlorophyll content was calculated using the formula described by [12] as follows:

Chl a (mg/ml) = 11.64 (A663) – 2.16 (A645)
Chl b (mg/ml) = 20.97 (A645) – 3.94 (A663)

Where:

A663 is the optical density (absorbance) at wavelength 663nm for chlorophyll a

A645 is the optical density at wavelength 645nm for chlorophyll b

Dry weight of shoots and corms/roots: At the end of the experiment, dry weight of shoots and corms (including roots) were recorded. Samples were dried in an oven at 105°C for 72 h. Possible phytotoxic effects of BTH were also evaluated.

2.4 *In vitro* Effect of BTH on Mycelial Growth of *P. myriotylum*

To investigate an eventual direct antagonism of BTH on *P. myriotylum*, the effect of BTH on mycelial growth was assessed *in vitro* using PDA amended with two concentrations (15 mg/L and 50 mg/L). Non-amended PDA was used as control. BTH was incorporated into the PDA medium before autoclaving. One mycelial plug, 5 mm in diameter, was removed from actively growing culture (5-7 day old cultures) of *P. myriotylum* and placed fungus-side down in the centre of each 9 cm-PDA plate. The plates were incubated at 28ºC (optimal growth temperature of *P. myriotylum*) in an incubator. Each treatment had three replicates and the experiment was repeated three times.

Radial growth was assessed by measuring the diameter of fungal colonies in two perpendicular directions and calculating the mean diameter at 40 h after incubation which corresponded to the time taken for the colony to grow up to the edge of the medium in the control plates. The diameter of the plug (5 mm) was subtracted from the colony diameter before estimating mean diameter of the colony.
The *in vitro* percentage inhibition was calculated according to the formula:

$$\text{Percentage of inhibition} = ((C-T))/C \times 100$$

Where:

C = colony diameter (mm) of the control
T = colony diameter (mm) of the test plate

2.5 Data Analysis

Data were subjected to tests of analysis of variance (ANOVA). After rejecting null hypothesis of equal means using ANOVA F-test, Fisher's Least Significant Difference (L.S.D.) was used for comparing treatment group means at 0.05. Statistical software Genstat computer package 5 Release 3.2 was used for Windows. Values were means ± standard deviation.

3. RESULTS AND DISCUSSION

3.1 Effect of BTH on CRRD Severity

Chemical activation of disease resistance in plants could represent an additional option for crop protection. This may be a good option for controlling soil borne pathogens where chemical application is difficult. Resistance inducers may also provide a complementary option for chemical control against some pathogens where genetic resistance is not available or not sufficient. CRRD is a devastating disease and given the lack of effective control measures, corm treatment with BTH was evaluated as a preventive management strategy during early attack. BTH applied as corm treatment induced various levels of CRRD protection, as identified by disease index in plants inoculated with *P. myriotylum*. Disease symptoms were observed in all inoculated plants but greatly reduced in plants treated with BTH. The two tested levels of BTH were found to be effective in inducing statistically significant levels of resistance against *P. myriotylum*. This was expressed by a disease index percentage of 37 on the 28th day post-inoculation for the low concentration tested (15 mg/l). Disease index decreased to 20 with increase in concentration of BTH (Fig. 1). Plants from control corms sown in inoculated soil showed the highest disease index percentage. In comparison, seed soaking in solutions of BTH at 25 or 50 mg/L consistently reduced disease severity of black root rot caused by *Thielaviopsis basicola* in cotton seedlings [13]. Applications of BTH have been shown to be effective in decreasing susceptibility in several plant species [14-16].

In earlier study, [6] showed that foliar application of BTH stimulated defence reactions in *X.*

sagittifolium. The resistance was noted as a decrease in CRRD incidence and severity. However, BTH application as corm treatment, as described in this study, suggests an easy, rapid and cheaper procedure for obtaining maximum protection against CRRD during sprouting, emergence and the early establishment phase of the crop. Using a combination of these two modes of BTH application may offer a more effective approach in managing CRRD.

Although BTH did not completely suppress the CRRD, the reduction and delay of symptoms by this elicitor could be exploited by giving possibility to cocoyam growers to apply and combine other CRRD control methods such as the use of tolerant varieties and composts.

The fungicide also significantly reduced CRRD when applied as corm treatment with a disease index percentage of 36. But BTH at 50 mg/L was significantly more effective than the fungicide. BTH is considered an environmentally friendly crop protection agent and plant defence activator and has been tentatively employed to reduce the use of agrotoxic substances while maintaining crop yields [17]. The results presented here showed that exogenously applied BTH to corm provided protection to cocoyam plant similar or better to that of the standard fungicide. These observations shed more light on the potential of BTH as a valuable alternative means of CRRD control.

3.2 Effect of BTH on Cocoyam Vegetative Growth

The effect of BTH on cocoyam growth is presented in the Table 1. Sprouting was observed in all the cocoyam corms soaked in different treatments. The chemicals tested affected only the time to sprouting. Sprouting time was significantly increased in corms treated with 15 mg/L of BTH as compared to control ones. Corms treated with 50 mg/L of BTH or with the fungicide showed sprouting time of 8.34 and 8.63 days respectively. No evidence of phytotoxic symptoms in terms of disruption of plant growth, leaf stunting or scorching was observed within days following all treatment application.

There was no significant difference in the chlorophyll content of the leaves between means of treated and control plants. But shoot dry weight of plants treated with 50 mg/L BTH was significantly reduced when compared with fungicide treated plants. BTH significantly reduced the dry weight of corms/roots as compared to the control.

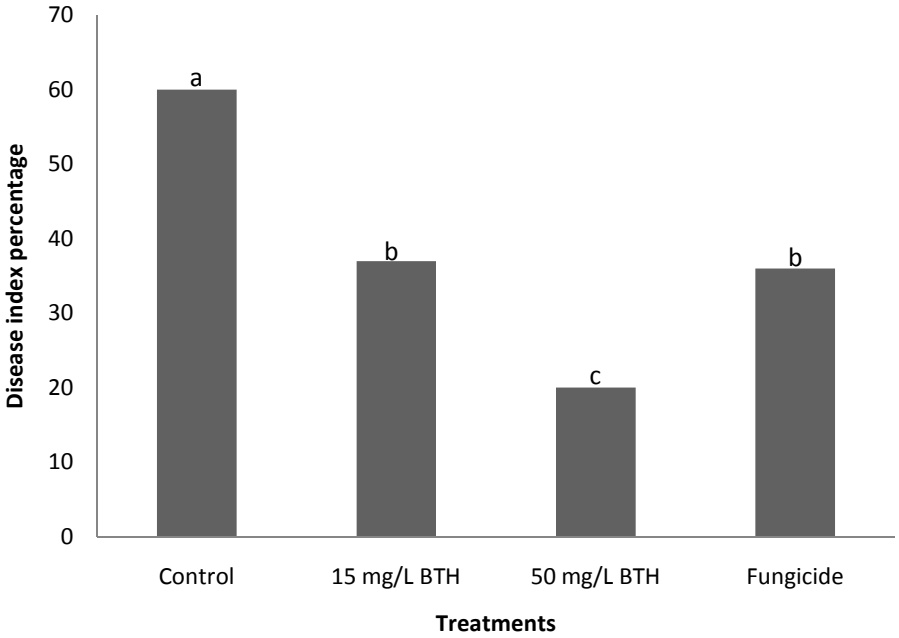

Fig. 1. Effect of corm treatment with BTH to control *P. myriotylum* in cocoyam
Bars with common letter do not differ significantly according to Fisher's L.S.D.

The deleterious effects were only observed as reduced growth but not foliar damage or reduced chlorophyll content; therefore it might be possible to overcome the reductions in dry mass via fertilization. The negative influence of BTH on plant growth is well documented and has been explained as a consequence of the allocation cost arising from the metabolic competition between the processes involved in plant growth and the de novo synthesis of defence-related compounds [18]. Thus it is likely that BTH controls root rot by activating the defence responses rather than by directly affecting root development and function. Such growth defects triggered by BTH may also depend on abiotic conditions [19] and host genetics, since some plants are much more sensitive to BTH-induced toxicity than others [20]. These findings of negative impacts of BTH applications on the vegetative growth of cocoyam plants are consistent with previous study on bean and cucumber in which foliar spray of BTH led to adverse effects on growth [21].

3.3 Effect of BTH on *In vitro* Mycelial Growth of *P. myriotylum*

To address whether BTH has a direct action on *P. myriotylum*, possibly acting as a fungicidal compound, we checked if mycelial growth was affected. BTH did not reduce mycelial growth at 15 mg/L after 40 h of culture. But the *in vitro* growth of *P. myriotylum* depended partially on the concentration of BTH tested, because by increasing the concentration from 15 to 50 mg/L, growth of the pathogen was inhibited by only 12% which is not significant. These results confirmed that BTH possessed little or no direct antimicrobial activity when used at lower concentrations. Its protective effect against pathogen was mainly due to the induction of the plant's own defence mechanisms [22].

Although BTH at low concentration (15 mg/L) did not exhibit any fungitoxicity against *P. myriotylum* in the form of reduced mycelia growth, when applied to the corm at the same concentration, BTH reduced CRRD severity on inoculated plants. The induction of the defence mechanisms without the antifungal activity was enough to suppress the infection in cocoyam plants treated with BTH and inoculated with *P. myriotylum*. These results showed that the protective effect of BTH against CRRD could only due to its host defence-inducing activity not to adverse effect on growth of the pathogen. This was also demonstrated in our study by temporal separation of the inducer and the pathogen inoculation. Induced systemic resistance normally requires a time-lapse period between inducer treatment and pathogen inoculation for gene activation of defense responses to take place [23].

4. CONCLUSION

Based on the present work, the use of BTH was efficient in protecting greenhouse-grown cocoyam plants against *P. myriotylum*, in a way similar to the standard fungicide. The protective effect of BTH cannot be attributed to the toxic effect of the chemical on the fungus, as the product did not show fungistatic activity against *P. myriotylum in vitro*. This work also showed that in the absence of pathogen, treated plants experienced reduced growth. Additionally, neither the BTH nor the fungicide treatments affected chlorophyll content in cocoyam. If confirmed in field tests, resistance induced by BTH has potential for control CRRD possibly in combination with existing crop protection practices.

Table 1. Effect of BTH corm treatment on growth parameters in cocoyam

Treatment	Sprouting time (days)	Chlorophyll content (mg/ml)	Shoot dry weight (g)	Corm/root dry weight (g)
Control	7.06 b	30.39 a	5.72 ab	8.42 a
15mg/L BTH	9.14 a	30.35 a	5.82 ab	6.02 b
50mg/L BTH	8.34 ab	29.49 a	4.62 b	4.96 b
Fungicide	8.63 ab	30.35 a	7.26 a	7.51 ab
LSD	2	2	2	2

In each column, means with similar letter(s) are not significantly different from each other Bars with common letter do not differ significantly according to Fisher's L.S.D.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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