

The production of gibberellin-like substances by *Colletotrichum gloeosporioides* associated with lupin anthracnose

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The most characteristic symptoms of lupin (*Lupinus* spp.) anthracnose caused by the fungus *Colletotrichum gloeosporioides*, are twisting and bending of stems and petioles. These might be related to the production of gibberellin-like activity by the pathogen. The barley aleurone bioassay was used to determine whether two isolates of *C. gloeosporioides* could produce gibberellin-like substances. Ethyl acetate extracts from iso-

lates SHK 2148 and SHK 1033, grown in liquid minimal medium, had gibberellin-like activities of 0.21 µg GA₃ equivalents and >0.26 µg GA₃ equivalents per g dry mass of mycelium, respectively. Provided that the strong association between gibberellins and this specific pathogen/host interaction can be established, this knowledge may be helpful in screening lupin cultivars for anthracnose resistance.

Introduction

Hormones such as auxins, cytokinins, ethylene, abscisic acid and gibberellins may act synergistically, antagonistically or additively to regulate plant growth and development (Graebe and Ropers 1978). In some cases, pathogen infection may elicit specific responses such as changes in leaf or stem morphology, abnormal root or flower development, or tumour production. Such responses usually indicate specific interference with growth regulation of the plant by metabolites produced by the pathogen (Von Anandel and Fuchs 1972).

Gibberellins are a group of natural plant hormones that have various effects on the growth and morphogenesis of plants (Pharis and King 1985). Gibberellin production has been extensively studied in *Gibberella fujikuroi* (Sawada) Wollenw. (Anamorph: *Fusarium fujikuroi* Nirenburg [G] mating population C in the *G. fujikuroi* species complex), and in cultures of other *Fusarium* species and several bacteria (Von Anandel and Fuchs 1972). The production of gibberellins in liquid cultures of *G. fujikuroi* was first reported by Kurosawa (1926). Production of gibberellins by *Sphaceloma manihicola* (Rademacher and Graebe 1979), *Neurospora crassa* Shear and Dodge (Kawanabe *et al.* 1983) (as cited by Tudzynski *et al.* 1998) and more recently by *Phaeosphaeria* spp. Cesati and de Notaris L487 (Kawaide and Sassa 1993) has been reported.

Lupin anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (Weimer 1943, Sweetingham *et al.* 1995, Reddy *et al.* 1996) is a devastating disease (Agaev 1993, Savchenko *et al.* 1994, Paulitz *et al.* 1995, Sweetingham *et al.* 1995, Frenzel 1998, Von Baer *et al.*

1999). Despite the seriousness of the disease, biochemical and physiological aspects involved in the infection process have not been investigated to any significant degree. The main symptoms of the disease are lupin plants displaying bent and twisted stems and dark brown, elongated lesions in the crook of the bend (Murray 1996, 1997). Diseased stems collapse, displaying characteristic tissue necrosis and crook-shaped distortion (Frenzel 1998). Lesions also develop on leaves and pods (Murray 1996). This phenomenon prompted us to investigate the possibility of a growth hormone being responsible for distortions which appear before lesion formation. The objective of the present study was therefore to ascertain whether *C. gloeosporioides* associated with lupin anthracnose produces a gibberellin-like substance(s) *in vitro*.

Materials and Methods

To determine whether the *Colletotrichum* isolates associated with lupin anthracnose produce gibberellin-like substances, the bioassay described by Jones and Varner (1967) was used with little modification. The conversion factors (CF) for the starch sample were first determined at 20°C and 25°C respectively by using the following equation:

$$CF = \mu\text{g } \alpha\text{-amylase} \times t \times v / \Delta\text{OD} \times T_v$$

where T_v = total volume of supernatant, ΔOD = OD of zero time (control) – OD of reaction sample, CF = conversion factor for starch sample, t = time of incubation and v = volume of supernatant taken for incubation. A standard curve relat-

ing gibberellic acid (GA₃) concentration and α -amylase released from barley half-seeds was created to determine activities of gibberellin-like substances produced by the test fungi.

Gibberellin extraction

Two single-spore isolates of *C. gloeosporioides* (SHK 1033 and SHK 2148) obtained from diseased lupin plants were grown for ten days in liquid minimal medium (2g NaNO₃, 30g sucrose, 1g KH₂PO₄, 0.5g MgSO₄·7H₂O, 0.5g KCl, 1 000ml distilled H₂O supplemented with 0.2ml trace element solution) in 250ml Erlenmeyer flasks. Ten flasks containing 150ml of medium were used for each isolate. One flask from SHK 1033 was rendered void due to contamination. The trace element solution consisted of 5g citric acid, 5g ZnSO₄, 1g Fe(NH₄)₂(SO₄)₂·6H₂O, 250mg CuSO₄·5H₂O, 50mg MnSO₄, 50mg boric acid, 50mg Na₂MoO₄·2H₂O and 95ml distilled water. Cultures were continuously shaken except for the first day after inoculation of flasks. After 10 days, the mycelial mat was harvested on Whatman No. 1 filter paper and the filtrate retained. The harvested mycelium was dried at 70°C for 48h and weighed. The pH of the filtrate was adjusted to 2.5 using 1N HCl. The gibberellin-like substance(s) was extracted with ethyl acetate from the filtrate using a separation funnel. The ethyl acetate fraction was dried using anhydrous Na₂SO₄ and concentrated at 45°C using a Buchi Rotavapor-R under vacuum. The ethyl acetate fraction was then subsequently dissolved in an acetate buffer solution (50ml of 3.8mM acetic acid adjusted to pH 4.8 using 4mM sodium acetate solution) to a final volume of 4ml. The solution was centrifuged for 10min at 2 000xg at 4°C. This solution was then used in the bioassay to detect gibberellin-like activity (Groenewald and Grobbelaar 1978).

α -amylase assay

A starch solution for the α -amylase assay was prepared from 150mg of potato soluble starch (Sigma S2630), 600mg KH₂PO₄ and 3mg calcium chloride in a total volume of 100ml distilled water. The mixture was boiled for 1min and then cooled before the clear supernatant was decanted off and used for the assay. An iodine stock solution was prepared by mixing 6g of potassium iodide and 600mg of iodine in 100ml of sterile distilled H₂O. One ml of the stock solution was added to 0.05N hydrochloric acid to give a final volume of 100ml. This was used to stop the α -amylase reaction.

Two-year-old barley seed (*Hordeum vulgare* L. cv. Chariot) was dehusked by treating with 50% sulphuric acid for 1h. The acid was decanted and the seed was washed thoroughly in distilled H₂O and air-dried overnight (Reeve and Crozier 1973). Seeds were cut in half transversely and the embryo portion discarded. The halved endosperms were disinfected by soaking in 1% NaOCl for twenty minutes, followed by three consecutive rinses in sterile H₂O. Disinfected seeds were imbibed on sterile sand in 100mm glass Petri dishes each containing 100g sand, moistened with 20ml sterile H₂O and placed in an incubator at 25°C. After a three day imbibing period, ten halved-seeds were transferred to 25ml Erlenmeyer flasks containing 0.5ml of acetate buffer

(pH 4.8), 0.2ml of 132mM of CaCl₂, and 0.2ml of the standard GA₃ solution or culture filtrate in a final volume of 2ml with distilled H₂O. Distilled H₂O was added to control treatments instead of the filtrate. As an added safety measure against microbial contamination, 0.2ml of 310 μ M chloramphenicol was added to each flask. Flasks containing the CaCl₂ and buffer were autoclaved prior to the start of the incubation period. Incubation of the halved seeds in the test solution was continued for 24h at room temperature with continuous shaking at 40 oscillations/minute. Following incubation, the contents were decanted into 100mm x 7.5mm centrifuge tubes and each flask was thoroughly washed three times, each time with 1ml of distilled H₂O. The washing liquid was added to the respective tube. The tubes were then centrifuged for 10min at 2 000xg at 4°C. The clarified supernatant solution was decanted and used for the assay.

The assay for α -amylase was accomplished using suitable volumes (0.02–0.2ml) of the supernatant fraction of 5 μ g ml⁻¹ α -amylase and sufficient water to make a total volume of 1.0ml. The chemical reaction was started by adding 1.0ml of starch solution or suspension to the medium and allowed to continue for a suitable period of time (1–10min). The reaction was stopped by the addition of 1.0ml of iodine reagent. To this final reaction mixture, 5.0ml of distilled H₂O was added, shaken and the optical density (OD) determined at 620nm by means of a Spectronic 20 spectrophotometer. OD values were converted to the amount (μ g) of α -amylase released, using a factor that was obtained for the particular starch sample by incubation with pure α -amylase. The concentration of α -amylase was calculated according to the following equation:

$$\alpha\text{-amylase } (\mu\text{g}) = \Delta\text{OD} \times T_v \times \text{CF}/t \times v$$

Results

The incubation time was determined using the pure α -amylase assay. According to Jones and Varner (1967), the decrease in OD at 620nm is directly proportional to the quantity of α -amylase present in the reaction mixture. The most accurate result is obtained by adjusting the α -amylase enzyme aliquot and time (t) so that the optical density of the starch-iodine complex is about 50% that of the zero time control. Based on this, the incubation period for the trials was set to be 3min at 20°C and 5min at 25°C (Table 1).

A nearly straight line relating α -amylase production with GA₃ concentration (Figure 1) was used as a standard curve to determine the amount of gibberellin-like substances produced by the fungal isolates.

Both fungal filtrates activated the secretion of α -amylase in halved barley seed (Table 2). Gibberellin-like secretions were determined to be 0.22 μ g and >0.26 μ g GA₃ equivalents per g dry mass of mycelium for isolates SHK 2148 and SHK 1033, respectively (Table 3). However, a very small amount of α -amylase was produced in the control treatments.

Discussion

The quantification of α -amylase released from barley half-seeds provides a reliable method for the determination of gibberellin-like substances in liquid culture (Jones and

Table 1: Pure α -amylase assay for the determination of the conversion factor (CF) from starch samples at incubation temperatures of 20°C and 25°C for 5min and 3min, respectively

20°C				25°C			
Zero OD (0min) ^d	Final OD (5min)	Δ OD	CF ^a	Zero OD ^b (0min)	Final OD ^c (3min)	Δ OD	CF
0.70	0.28	0.42	0.0119	0.95	0.44	0.51	0.0059
0.75	0.32	0.43	0.0116	0.90	0.38	0.52	0.0058
0.70	0.29	0.41	0.0122	0.95	0.37	0.58	0.0052
0.75	0.27	0.48	0.0104	0.95	0.48	0.47	0.0064
0.75	0.28	0.47	0.0106	0.90	0.38	0.52	0.0058
0.70	0.36	0.34	0.0147				
0.70	0.32	0.38	0.0132				
0.70	0.30	0.40	0.0125				
Average			0.0120				0.0060

Note: 0.2ml α -amylase (supernatant) at a concentration of 5mg ml⁻¹ and 8ml of T_v were used for assaying

^a Conversion factor (CF) = μ g α -amylase \times t \times v/ Δ OD \times T_v where T_v = total volume of supernatant, Δ OD = OD of zero time (control) – OD of reaction sample, CF = conversion factor for starch sample, t = time of incubation and v = volume of supernatant taken for incubation

^b Optical density reading at zero time control

^c Optical density reading following incubation

^d Incubation periods in minutes

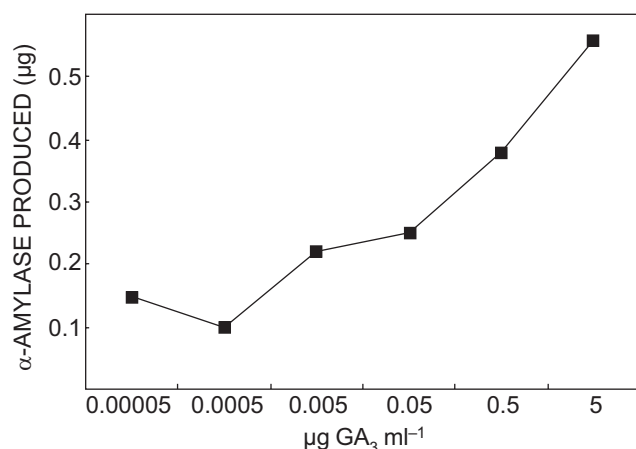


Figure 1: Standard curve indicating relationship between gibberellic acid (GA₃) concentration and α -amylase release from barley half-seeds

Varner 1967). This is despite the fact that not all barley cultivars are suitable for use as there is considerable variation among varieties in their sensitivity to GA₃. In addition, sensitivity is associated with season, area of growth and age of the seed. The barley aleurone bioassay is sensitive to GA₃ in doses as low as 0.001 μ g per flask (Reeve and Crozier 1973) or even 9pg of GA₃ (Salisbury and Ross 1992). The barley aleurone layer is well established as a model for studying hormonal regulation (Hambata *et al.* 1994). A characteristic of the aleurone layer of cereal seeds is its capacity to secrete a wide spectrum of hydrolytic enzymes, which play a role in endosperm degradation during germination (Moll and Jones 1982). In barley aleurone, the synthesis of many enzymes is controlled by gibberellin, calcium and abscisic acid (Hillmer *et al.* 1992). GA₃ induces the synthesis of α -amylase and several other hydrolytic enzymes (Ho

1991, Jacobsen and Close 1991). Protoplasts isolated from aleurone layers of barley responded to externally applied gibberellic acid with increased synthesis and the secretion of α -amylase (Gilroy and Jones 1994). The quantity of α -amylase produced is proportional to the logarithm of gibberellin concentration applied (Jones and Varner 1967).

Cytochemical and metabolic evidence has shown that the release of α -amylase from the halved barley is dependent on metabolic linked processes and not diffusion limited (Moll and Jones 1982). Heat shock imposed by raising the temperature of incubation from 25°C to 40°C, inhibited or suppressed the accumulation of α -amylase and other proteins in the incubation medium of barley aleurone layers treated with GA and Ca²⁺ (Brodl *et al.* 1990, Sticher *et al.* 1990, Lanciloti *et al.* 1996). Different responses in terms of α -amylase released from the two trials in this experiment were obtained because they were made at two different temperatures. The concentration of α -amylase released from incubation at 25°C was higher compared to that released from incubation at 20°C (Table 2). This finding is consistent with a report by Agu and Palmer (1997) where a malting temperature of 30°C produced more α -amylase than one of 20°C.

Despite great care being taken to eliminate GA₃ already present in the husk of the seed using sulphuric acid, very small amounts of α -amylase were still produced in the control treatment. Some reports associate α -amylase production with acidification of the starchy endosperm by aleurone cells, but in the present experiment, the pH of the incubation medium was not monitored. Hambata *et al.* (1988) and MacNicol and Jacobsen (1992) demonstrated that acidification of the endosperm was produced by the aleurone cells *per se* independent of the embryo or gibberellin present. Sinjorgo *et al.* (1993) reported increased activity of α -amylase and glyoxysomal isocitrate lyase (ICL) independent of gibberellic acid at pH 3.2, although the effect was most pronounced for ICL. At higher extracellular pH values, hormone induced enzyme activity decreased in a dose-dependent manner.

Table 2: Average change in optical density (OD) following the effect of GA₃-concentration on α -amylase released by barley seed incubated at 20°C and 25°C for 5min and 3min, respectively

Treatment	20°C					25°C				
	TVS ^a	Zero OD ^b	Final OD ^c	Δ OD ^d	α -amylase ^e (μ g)	TVS	Zero OD	Final OD	Δ OD	α -amylase (μ g)
5 x 10 ⁻⁴ μ g GA ₃	5.4	0.63	0.52	0.11	0.10	5.0	0.78	0.69	0.09	0.11
5 x 10 ⁻³ μ g GA ₃	6.1	0.54	0.43	0.11	0.19	6.1	0.76	0.59	0.17	0.25
5 x 10 ⁻² μ g GA ₃	3.5	0.27	0.22	0.05	0.10	6.2	0.75	0.47	0.28	0.42
5 x 10 ⁻¹ μ g GA ₃	5.4	0.58	0.39	0.19	0.31	5.4	0.76	0.42	0.34	0.44
SHK 2148	5.3	0.51	0.31	0.20	0.32	5.0	0.71	0.32	0.39	0.47
SHK 1033	5.3	0.47	0.26	0.21	0.33	5.8	0.73	0.29	0.44	0.61
Control	5.2	0.68	0.64	0.04	0.06	5.5	0.94	0.94	0.01	0.03

Note: Conversion factors 0.30 and 0.14 were used to calculate α -amylase level produced at 20°C and 25°C for 5min and 3min incubation periods respectively; 0.2ml was the volume of supernatant taken for incubation

^a Total volume of supernatant

^b Optical density reading at zero time control

^c Optical density reading following incubation

^d The difference between optical density reading at zero time control and optical density reading following incubation

^e α -amylase (μ g) = Δ OD x T_v x CF/t x v

Table 3: Total dry mass of mycelium (g), average μ g α -amylase produced, and the extrapolated average GA₃-equivalents (μ g g⁻¹) of dry mycelium for two isolates of *C. gloeosporioides*

Isolate No.	TDMW ^a	LMM ^b (g)	α -amylase (l)	GA ₃ -like substance(s) (μ g)	GA ₃ equivalent (μ g) per g mycelial dry mass
SHK 2148 ^c	23.13	1.35	0.40	0.500	0.22
SHK 1033	19.18	1.50	0.46	>0.500	>0.26
Control	0.00	1.50	0.04	<0.005	

^a Total dry mycelium weight

^b Liquid minimal medium used in litres

^c Derived from Standard Curve (Figure 1)

Some evidence is available that GA₃-producing microorganisms often induce harmful reactions in host plants. In isolates of *F. fujikuroi*, the causal agent of bakanae disease of rice, a positive correlation existed between the biosynthesis of GA₃ *in vitro* and symptom expression (Sunder *et al.* 1998). In *Sorghum bicolor* (L.) Moench, the application of GA₃ reversed floral primordials to vegetative leafy growth (phylloidy), which is a common symptom associated with sorghum head smut. It was concluded that phylloidy in partially infected sorghum plants could be due to an increase in GA₃ in affected plants (Bhaskaran *et al.* 1990). Gibberellin found in the extract of the fungal culture medium promoted shoot elongation in sorghum (Beall *et al.* 1991). Matheussen *et al.* (1991) noted a reduction in early 13-hydroxylation precursors in all the smutted or sterile panicle samples compared to their appropriate controls. This reduction suggests that this pathway is blocked or that precursors are diverted to other uses by the fungus (*Sporisorium reilianum*), although the fungus can produce GA₁ and GA₃ when cultured on Richard's medium. The result could be due to a deficiency of bioactive gibberellins at the time of internode elongation.

Reports also indicate that exogenously applied abscisic acid or gibberellic acid increased the susceptibility of non-host plants to cowpea rust (Li and Heath 1990a). Intercellular washing fluids obtained from rust-infected bean

(*Phaseolus vulgaris* L.) leaves and other compatible rust-plant interactions also suppressed silica deposition in bean and increased the incidence of haustoria subsequently produced by the cowpea rust fungus (Heath 1979, 1981). In contrast, however, Li and Heath (1990b) did not see any effect using intercellular washing. In cowpea rust, the absence of haustoria in non-host interactions is associated with the presence of silica deposits on the plant wall (Heath 1979, Stumpf and Heath 1985). Earlier, Soni *et al.* (1972) reported that silica deposition is regulated by gibberellic acid in *Avena* epidermal cells. Injection of both abscisic acid and gibberellic acid resulted in decreased silica deposition and autofluorescent walls in mesophyll cells (Stumpf and Heath 1985, Li and Heath 1990a) and increased the incidence of haustoria (Li and Heath 1990a). A similar effect was also observed for the corn rust fungus, except that the incidence of autofluorescent walls was not reduced, probably because of the increased autofluorescence of the contents of invaded cells (Li and Heath 1990a).

The present study represents the first time that the production of gibberellin-like substances has been associated with any *Colletotrichum* spp. Preliminary results of the present study suggest that the detection of gibberellin-like substances from pure cultures of *C. gloeosporioides* grown in minimal liquid medium could pave the way for assessing its effect in host pathogenesis. Bending and twisting of the

stems and petioles, and deformation of pods and leaves of lupins infected by *C. gloeosporioides* even before lesions are visible, may be related to GA₃ production by the pathogen as gibberellins stimulate cell division, and promote cell growth and cell-wall plasticity (Salisbury and Ross 1992). These authors speculated that the sugars released in the host plant by hydrolase enzymes induced by gibberellins might serve as an energy source for the fungus.

Sunder *et al.* (1998) suggested that prior knowledge of the ability of *F. fujikoroii* isolates from *bakanae* disease of rice to produce more GA₃ may help in selecting isolates for testing disease resistance. Similarly, if increased GA₃ production in lupins can be correlated with increased severity of symptoms in the *C. gloeosporioides*–lupin interaction, this knowledge may be helpful in screening cultivars for anthracnose resistance provided that the association is established experimentally.

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