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The effect of potential resistance inducers on development of *Microdochium majus* and *Fusarium culmorum* in winter wheat

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Abstract The effect of potential resistance inducing chemicals on disease development of *Fusarium* head blight was studied in winter wheat (*Triticum aestivum* L.). As a pre-screening test, the effect of different treatments on development of *Microdochium majus* (syn. *Microdochium nivale* var. *majus*) was studied in detached leaves. Based on these tests, DL-3-aminobutyric acid, Bion (benzo-(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester), and a foliar fertilizer containing potassium phosphite were selected for further studies. Greenhouse-grown winter wheat was sprayed with aqueous solutions of the potential resistance inducers 7 days prior to *Fusarium culmorum* point inoculation of the heads. Disease development was registered as number of bleached spikelets per inoculated spike. Spraying plants with the foliar fertilizer reduced the disease severity of *F. culmorum* by up to 40%. A reduced disease development of *M. majus* was also observed in detached leaves pre-treated with the foliar fertilizer. When the foliar fertilizer was added to the growth medium, a reduced in vitro growth of *M. majus* and *F. culmorum* was observed, indicating that the effect on disease development is at least partly due to a fungistatic effect. No significant reduction in disease development was observed in wheat pre-treated with DL-3-aminobutyric acid or Bion, although these compounds tended to reduce disease development, especially when applied in combination with other potential resistance inducers. We conclude that spraying winter wheat with a solution containing potassium phosphite can reduce development of *M. majus* and *F. culmorum*.

Keywords Bion · Detached leaf assay · DL-3-aminobutyric acid · Induced resistance · Phosponate · Potassium phosphite

Abbreviations

BABA	DL-3-aminobutyric acid
BTH	benzo-(1,2,3) thiadiazole-7-carbothioic acid-S-methyl ester
FHB	*Fusarium* head blight
ISR	induced systemic resistance

Introduction

Fusarium head blight (FHB) is a widespread and destructive disease of cereals caused by a number of *Fusarium* species. *Microdochium majus* (Wollenw.)

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Glynn & S.G. Edwards (Glynn et al. 2005) (syn. *Microdochium nivale* var. *majus*) is also part of the complex causing head blight symptoms in cereals. FHB can reduce grain quality due to the production of a range of toxic metabolites that have adverse effect on humans and livestock. Due to the lack of consistently effective control measures, FHB poses a significant threat to the yield and quality of small grained cereals (Pirgozliev et al. 2003).

Plants seem to constantly adjust their defensive status to the dynamic changing environment (van Loon et al. 2006). Induced disease resistance seems to constitute a mechanism through which the level of general resistance to pathogens is increased (van Loon 1997). In *Arabidopsis* it has been discussed whether effective defence against biotrophic pathogens can be due to activation of defence responses regulated by the salicylic acid-dependent pathway, whereas defence against necrotrophic pathogens are limited by a different set of defence responses activated by jasmonic acid and ethylene signalling (Glazebrook 2005). According to Tuzun and Bent (2006), induced systemic resistance (ISR) is the correct term to describe activated defence mechanisms whether the inducers are pathogenic or non-pathogenic organisms or chemicals. It has been proposed that the different variants of induced resistance should be distinguished according to the pathway they activate: salicylate-dependent ISR (SA-ISR) or jasmonate-dependent ISR (JA-ISR) (Tuzun and Bent 2006).

In wheat, induction of defence responses has been registered after pathogen attack, after treatment with fungal cell wall fractions and after treatment with several resistance inducers (Caruso et al. 1999; Bertini et al. 2003; Kuwabara et al. 2002; Oka and Cohen 2001; Görlach et al. 1996). Enhanced expression of genes often associated with induced disease resistance might also play a role in resistance to *Fusarium* spp. in wheat (Li et al. 2001; Mackintosh et al. 2007; Desmond et al. 2005).

The aim of these studies was to identify a resistance inducer treatment with potential to reduce development of FHB in winter wheat. We intended to reveal whether a detached leaf assay could be used as a screening to select treatments for potential FHB control. Several putative resistance inducer agents including chemicals, elicitors and plant and fungal extracts were pre-screened for their ability to reduce disease development of *M. majus* in detached leaves of winter wheat. Selected compounds were further tested in a controlled environment for their capacity to reduce development of *Fusarium culmorum* in heads of winter wheat.

Materials and methods

Chemical compounds

Various putative resistance-inducing compounds including chemicals as well as plant and fungal extracts were screened for their ability to reduce symptom development of *M. majus* in detached leaves of winter wheat in experiments A–D (Table 1). After a pre-screen of the effect of the different treatments on *M. majus* development on detached leaves in experiment A (data not shown), BABA, Bion, a foliar fertilizer solution containing potassium phosphite (hereafter named 'PPS'), chitosan from Fluka (hereafter named 'chitosan F'), chitosan from Aldricht (hereafter named 'chitosan A') and Trehalose were selected for further use in experiment B–D (Table 1). The concentrations and the combinations of putative resistance inducers in experiments B–D were mainly based upon results from the pre-screening tests in experiment A. Three of these treatments (Bion, BABA, and PPS) were further evaluated for their effect on *F. culmorum* development in heads of winter wheat (experiment E, Table 1).

Microdochium majus, detached leaf assay

Development of *M. majus* was studied in detached leaves of winter wheat pre-treated with various potential resistance inducing chemicals (experiments A–D, Table 1). Winter wheat cultivar Bjørke (Svalöf Weibull AB, Svalöv, Sweden) was sown in 10 cm pots (five seeds per pot) containing a fertilized mixture of sand and peat (P-jord, Emmaljunga Torvmull AB, Emmaljunga, Sweden) and cultivated in a greenhouse at about 18/12°C day/night temperature and 16 h supplemental light (Philips HPI, Philips, Eindhoven, The Netherlands) at approximately 150 µmol photosynthetically active radiation (PAR) m^{-2} s^{-1}). After 2 weeks, the leaves of the seedlings were treated with aqueous solutions of the various putative resistance inducing compounds

Table 1 Some of the different treatments used in this study

Treatment		Manufacturer	Concentration	Experiments[a]
BABA	DL-3-aminobutyric acid, $CH_3CH(NH_2)CH_2CO_2H$ (Cat: A4, 420-7)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	100 μg ml^{-1}	A
			500 μg ml^{-1}	A
			1,000 μg ml^{-1}	A, E
			8,000 μg ml^{-1}	A, B, C, E
Bion	50% w/w BTH (1,2,3-benzothiadiazole-7-carbothioic acid S-methyl ester) syn.: Actigard, Syngenta	Novartis, Ltd., Basel, Switzerland,	10 μg ml^{-1}	A, E
			50 μg ml^{-1}	A
			100 μg ml^{-1}	A, B, C, D, E
Chitosan (Aldricht)	Low molecular weight chitosan from Aldrich (cat. no. 44,886-9, 75–85% deacetylated, 20–200 cps (1% solution in 1% acetic acid)	Aldrich (Milwaukee, WI, USA)	100 μg ml^{-1}	A, B, C
			500 μg ml^{-1}	A
			1,000 μg ml^{-1}	A
Chitosan (Fluka)	Low molecular weight chitosan prod. No. 22741, viscosity 100 mPas (1% solution in 1% acetic acid))	Fluka Chemie AG, Buchs, Switzerland	100 μg ml^{-1}	A
			500 μg ml^{-1}	A
			1,000 μg ml^{-1}	A, B, C
Chitosan (211)	Chitosan CL 211 (no. 211-490-06, 50% deacetylated, viscosity of 70 mPa.s. in 1% solution)	Protan Biopolymers A/S, Norway,	100 μg ml^{-1}	A
			500 μg ml^{-1}	A
			1,000 μg ml^{-1}	A
Milsana	Milsana Flüssig	Biofa GmbH, Münsingen, Germany	15 μl ml^{-1}	A
Potassium phosphite solution ('PPS')	A foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution (K_2HPO_3) in addition to small quantities of amino acids, anti foam agents, polyols, vitamins and wetting agents (solution density 1.23 g/ml at 16C)	Taminco, UK	1 μl ml^{-1}	A, C, E
			5 μl ml^{-1}	B
			10 μl ml^{-1}	A, B, D, E
			20 μl ml^{-1}, 30 μl ml^{-1}, 40 μl ml^{-1}, 50 μl ml^{-1}, 60 μl ml^{-1}, 70 μl ml^{-1}, 80 μl ml^{-1}, 90 μl ml^{-1}	D
			100 μl ml^{-1}	C, D, E
Trehalose	D-(+)-Trehalosedihydrate, EC No. 2027396	Fluka Chemie AG, Buchs, Switzerland	15 mg ml^{-1}	A
			30 mg ml^{-1}	A, B, C
TRF-AGRO	Organic complex compound, integrated mainly from citrus polyphenols: citrus bioflavonoids, ascorbic acid, citrus phytoalexins, citrus biomass	Trifolio-M GmbH, Lahnau, Germany	2.5 mg ml^{-1}	A
			5 mg ml^{-1}	A
TRF-MS	An extract from cell walls of specific fungi. Contains glucane, melanin, lipids, amino acids, proteins and mineral components	Trifolio-M GmbH, Lahnau, Germany	30 μl ml^{-1}	A
			50 μl ml^{-1}	A

[a] The treatment effects were studied on *Microdochium majus* inoculated wheat leaves (Experiments A–D) and in *Fusarium culmorum* inoculated heads of winter wheat (Experiment E)

added 0.01% Tween 20 (Fluka Chemie, Buchs, Switzerland). The solutions (0.5 ml per plant) were applied by using a high-pressure sprayer. Distilled water containing 0.01% Tween 20 was used as control treatments. One week after treatment, 5 cm segments from the mid-section of the second leaf were harvested and placed on petri dishes containing water agar (BD Bacto™ agar, Becton, Dickinson & Company, Le pont de Claix, France), 5 g l^{-1} and kinetin (Sigma-Aldrich, Steinheim, Germany), 10 mg l^{-1}. The petri dishes, each containing four leaf segments from separate plants, were incubated in a Termax chamber (Termax, Bergen, Norway) at 10–12°C at 12 h white light (OSRAM L36W/840 Lumilux cool white) at 150 µmol PAR m^{-2} s^{-1} in combination with 'black light' (OSRAM L36W/73, München, Germany). The leaf segments were inoculated the following day with a 10 µl droplet of a *M. majus* spore suspension adjusted to 1×10^6 conidia ml^{-1} distilled water containing 0.01% Tween 20. Spores were produced by inoculating a 5 mm plug of actively growing *M. majus* isolate OP2A onto a cellophane layer (Cellophane sheets, Sigma-Aldrich, Steinheim, Germany) on potato dextrose agar (PDA) in 9 cm petri dishes (Browne and Cooke 2004a). The leaf segments were further incubated in the Termax chamber with the same temperature and light quality as described above. Assessments of incubation period (days from inoculation until symptom appearance) and latent period (days from inoculation until sporulation) were recorded daily using a compound microscope, as described by Browne and Cooke 2004b. There were always four replicate petri dishes for each treatment within a replicate of an experiment.

Based upon results obtained from a pre-screening tests (experiment A) where several different potential resistance inducers were included (some of the compounds are presented in Table 1), six compounds were selected for further use in experiment B and C. Experiment C included the same six compounds as experiment B, but the concentrations for some of the compounds differed from those used in experiment B and the experiment was conducted once. To study the effect of photassium phosphite concentration on disease development and to further confirm the findings from experiment B and C, only PPS and Bion were tested in experiment D. Experiment D was conducted three times. In most experiments the compounds were applied alone and in a mixture with other compounds.

Fusarium culmorum, greenhouse studies

Selected treatments were tested in a controlled environment for their capacity to reduce development of *F. culmorum* in heads of winter wheat (Table 1, experiment E). Seeds of winter wheat were surface sterilized and placed onto wetted filter paper in 9 cm petri dishes. After 10 weeks of vernalization at 2–4°C, the seeds were transplanted to 19 cm pots (seven plants per pot) containing a fertilized mixture of sand and peat (P-jord, Emmaljunga Torvmull AB, Emmaljunga, Sweden). The seedlings were further grown in a greenhouse for 10 weeks at 15/10°C day/night temperature, 65% relative humidity and 16 h additional light (Philips HPI, at about 200 µmol PAR m^{-2} s^{-1}). During cultivation the plants were fertilized two times weekly with a nutrient solution composed of 'Superba rød' (7% N, 4% P, 21% K, HYDRO, Norway), potassium nitrate (kaliumnitrat, 13,7% N, 38,6% K, LOG, Norway), ammonium nitrate (Ammoniumnitrat, 34.5% N, LOG, Norway), and micronutrients (Pioner mikronæring, Brøste A/S, Lyngby, Danmark) adjusted to a final concentration of 104 mg N l^{-1}. At heading, 1 week prior to flowering, the plants were sprayed with aqueous solutions of Bion, BABA or the foliar fertilizer 'PPS' containing 0.01% Tween 20 (Table 1) by using a high-pressure sprayer (20 ml per pot). Distilled water containing 0.01% Tween 20 was used as control treatments. At flowering, single spikelets in six to ten heads within each pot were inoculated with a 10 µl droplet of a *F. culmorum* spore suspension by using a micropipette. The spore suspension was produced from *F. culmorum* colonies grown on PDA at 18–20°C and 12 h white light+NUV. After 3 weeks of growth, conidia were washed off the agar surface and diluted in distilled water containing 0.01% Tween 20 to a final concentration of 1×10^5 conidia ml^{-1}. In the first replicate of the experiment, a mixed solution of conidia from four different isolates of *F. culmorum* was used. In the last two replicates of the experiment the inoculum was made from only one *F. culmorum* isolate. After inoculation, the heads were covered with plastic bags for 48 h. The plants were further grown in a greenhouse at 75% relative humidity, 20°C day (12 h) and 15°C night (12 h) and 18 h additional light (Philips HPI, at about 200 µmol PAR m^{-2} s^{-1}). Disease symptoms were scored as number of bleached spikelets below the inoculation point in

each head at 11 days, 17 days and 23 days after inoculation. There were 13 treatments within each of the three replicate of the experiment and four replicate pots within each treatment.

In vitro fungal growth

The effect of photassium phosphite on in vitro growth of *M. majus*, *F. culmorum* and *F. graminearum* was measured. PPS was added to autoclaved PDA to final concentrations of 0 μl l^{-1}, 10 μl l^{-1}, 50 μl l^{-1}, 100 μl l^{-1}. The agar was thereafter poured into 9 cm dishes (20 ml $dish^{-1}$). The dishes were inoculated with agar plugs (5 mm) of actively growing mycelium and sealed with parafilm. The diameter of the fungal colony was measured after 3 days of growth in darkness at 20°C. The growth rate for each combination of fungal isolate and PPS concentration was calculated as the average daily radial growth of mycelium in three replicate dishes.

Statistical analysis

Average incubation period and latent periods within petri dish recorded in the detached leaf assays were subjected to statistical analysis. Within each replicate of the experiments B–D, there were four petri dishes of each treatment. Replicate was used as a factor in the statistical analysis. As input for the statistical analysis of in experiment C, latent period was set to 12 days for leaf segments that did not develop symptoms during the period of registration (up to 11 days after inoculation). Data on incubation period were not statistically analyzed in experiment C due to a lack of symptom development in these same leaves. In each replicate of experiment D, symptom development was registered until 3 days after sporulation was observed on more than 80% of the leaves in the water treated control (11 days, 9 days and 9 days after inoculation in the three replicates of the experiment, respectively). For the statistical analysis, latent periods of 12 days, 10 days and 10 days were set for leaves where no symptom development was observed during the period of registration in each of the three replicates of the experiment, respectively. Data on incubation period were not statistically analyzed in experiment D due to a lack of symptom development in these same leaves. Analysis of variance procedure general linear model (GLM) was performed using the SAS® (SAS Institute Inc., Cary, NC, USA) program for Windows (Release 9.1). Significant treatment differences were separated by the use of Dunett-Hsu LSD in SAS® at $P \leq 0.05$.

Average calculated numbers of bleached spikelets per inoculated spike within pot registered in the greenhouse experiments were used in the statistical analysis (GLM in SAS®). Each replicate of the experiment contained four pots within treatment and the experiment was conducted three times. Replicate was used as a factor in the statistical analysis. Significant treatment differences were separated by the use of Dunett LSD in SAS® at $P \leq 0.05$.

Results

Microdochium majus, detached leaf assay

Several treatments shortened the incubation period of *M. majus* on detached wheat leaves, as compared to water-treated control leaves in experiment B (Fig. 1a). This reduction in incubation period was significant in leaves pre-treated with solutions containing BABA (8,000 μg ml^{-1}) in combination with chitosan F at 1,000 μg ml^{-1} or Bion at 100 μg ml^{-1}. At inoculation, small chlorotic leaf spots were observed in one or both replicates of the experiment on leaves pre-treated with chitosan F at 1,000 μg ml^{-1} and on leaves pre-treated with BABA 8,000 μg ml^{-1} in combination with the following treatments: Chitosan A at 100 μg ml^{-1}, Chitosan F at 1,000 μg ml^{-1}, Trehalose at 30 mg ml^{-1} and PPS at 10 μl ml^{-1}. Enhanced latent periods (not significant) were observed on leaves from most treatments compared to the water-treated controls (Fig. 1b).

In experiment C, the onset of sporulation was delayed on most leaves pre-treated with putative resistance activators, compared to water treated control. At inoculation, small chlorotic leaf spots could be observed on leaves pre-treated with chitosan A at 100 μg ml^{-1} and on leaves pre-treated with BABA 8,000 μg ml^{-1} in combination with Chitosan F (1,000 μg ml^{-1}) or Trehalose (30 mg ml^{-1}). Necrotic leaf tips were observed in some of the leaves pre-treated with PPS at 100 μl ml^{-1}, and PPS (100 μl ml^{-1}) in combination with Bion (100 μg ml^{-1}). At 4 days after inoculation, from 0–40% of the leaves within a treatment was sporulating, compared to 50% sporula-

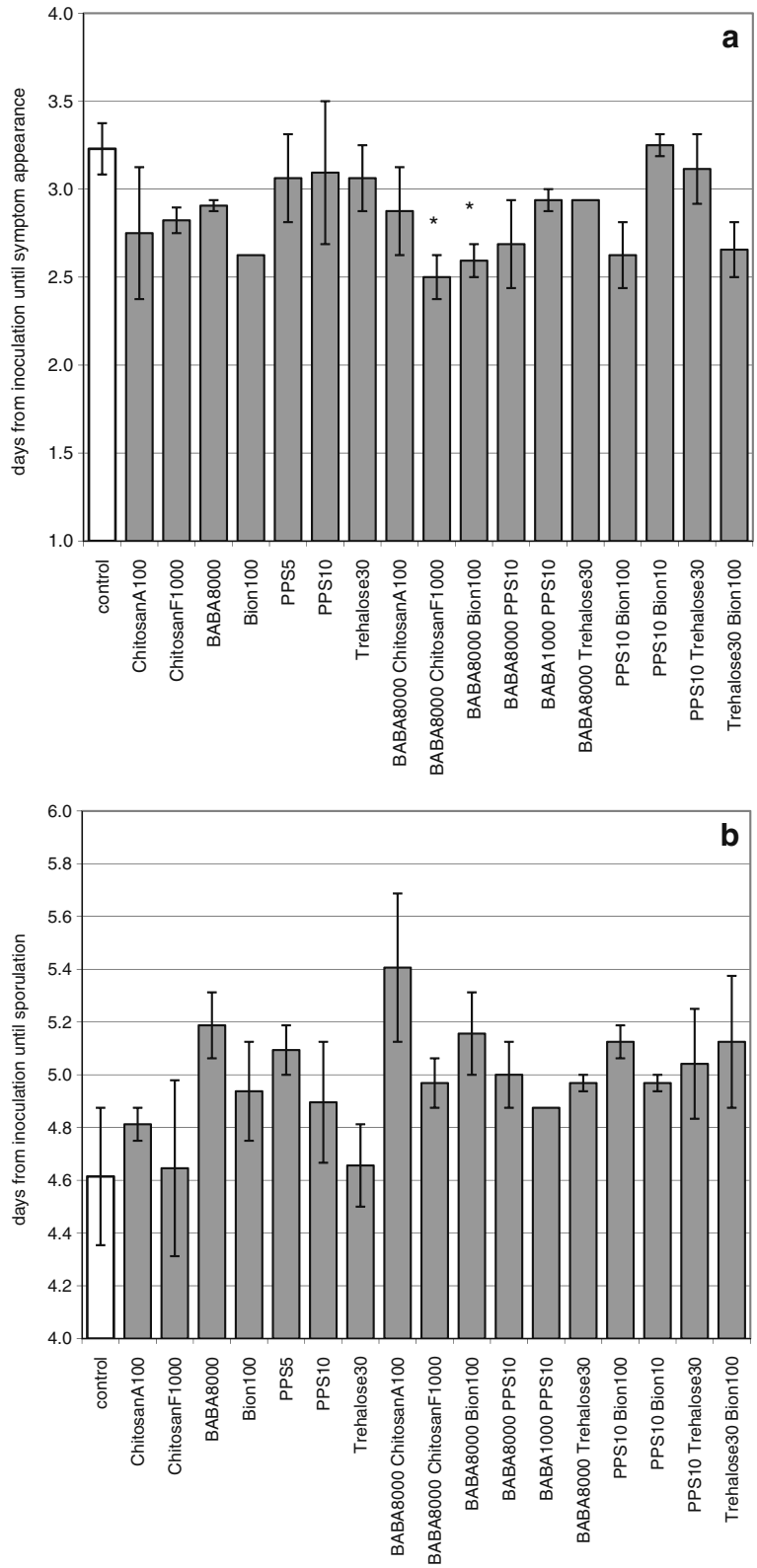

Fig. 1 The effect of potential resistance inducers on **a** symptom development (days from inoculation until symptom appearance, incubation period) and **b** sporulation (days from inoculation until sporulation, latent period) of *Microdochium majus* on detached leaves from 3-week old plants of winter wheat (Experiment B). The figure displays the following treatments: control = water, chitosanA100 = chitosan (Aldricht) at 100 μg ml^{-1}; chitosanF1000 = chitosan (Fluka) at 1,000 μg ml^{-1}; BABA8000 = DL-3-aminobutyric acid at 8,000 μg ml^{-1}; Bion100 = Bion at 100 μg ml^{-1}; PPS5 and PPS10 = 5 μl ml^{-1} or 10 μl ml^{-1} respectively, of a foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution; Trehalose30 = Trehalose at 30 mg ml^{-1}; Dewasid5 = Dewasid at 5 μl ml^{-1}. Several treatments consisted of a combination or the different putative resistance inducers. The plants were sprayed with the different aqueous solutions 1 week prior to leaf detachment. The detached leaves were incubated at 10°C and light, and inoculated the following day with a spore suspension of *M. majus*. The figure displays average values from two experiments (four replicate dishes within experiment). *Error bars* indicate standard errors of the mean. * = Treatment effects significantly different from the water control (GLM in SAS, Dunnett-Hsu, $p \leq 0.05$)

tion in the water treated control (Fig. 2). At 6 days after inoculation, 100% of the leaves were sporulating in most treatments except for PPS at 100 µl ml^{-1} or a combination of PPS (100 µl ml^{-1}) and Bion (100 µg ml^{-1}), where sporulation was observed in 88% and 81% of the leaves respectively. Although these two treatments tended to restrict sporulation, no significant difference in latent period was found.

In experiment D, the effect of different concentrations of PPS and Bion on development of *M. majus* was studied. At inoculation, necrotic leaf tips were observed in leaves pre-treated with PPS at 100 µl ml^{-1}, and in leaves pre-treated with PPS at 100 µl ml^{-1} in combination with Bion at 100 µg ml^{-1} for all tree replicates of the experiment. In two out of three replicates of the experiment, necrotic leaf tips were also observed in leaves from plants pre-treated with PPS at concentrations exceeding 60 µl ml^{-1}. Latent period increased with increasing concentration of PPS, and the latent period was significantly longer (Dunett-Hsu LSD in SAS® at $P \leq 0.05$) compared to water treated control at PPS concentrations of 50 µl ml^{-1}, 70 µl ml^{-1}, 80 µl ml^{-1}, 90 µl ml^{-1} and 100 µl ml^{-1} (Fig. 3a). The relationship between PPS concentration (X, µl ml^{-1}) and days to sporulation (Y) is presented in Fig. 3a according to the following equation:

$Y = 5,868 + 0,03721X - 0,000163X^2$, $r^2(adj) =$ 87,8%, p=0.000, F=33,3 (Polynomial Regression Analysis in MINITAB). A maximum delay in latent period of almost 2 days was registered in plants pre-treated with PPS at 90 µl ml^{-1}. A combination of PPS (100 µl ml^{-1}) and Bion (100 µg ml^{-1}) also gave a significant reduction in development of *M. majus* of detached leaves (Fig. 3b). No reduction in disease development was found on leaves pre-treated with a

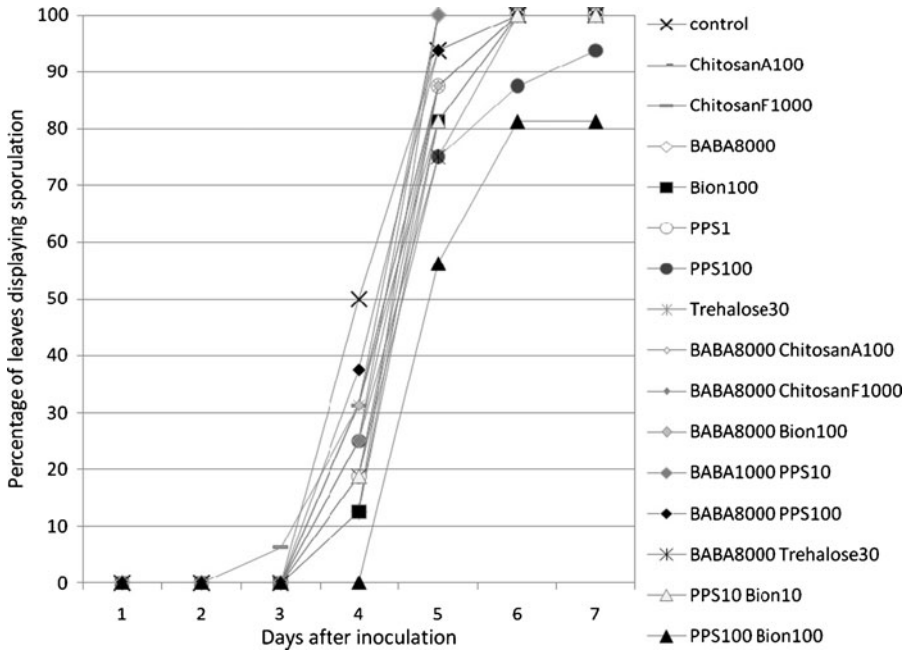

Fig. 2 The effect of potential resistance inducers on sporulation of *Microdochium majus* on detached leaves from 3-week old winter wheat (experiment C). Control = water; chitosanA100 = chitosan (from Aldricht) at 100 µg ml^{-1}; chitosanF1000 = chitosan (from Fluka) at 1,000 µg ml^{-1}; BABA8000 = DL-3-aminobutyric acid at 8,000 µg ml^{-1}; Bion100 = Bion at 100 µg ml^{-1}; PPS1 or PPS100 = 1 or 100 µl ml^{-1} respectively, of a foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution; Trehalose30 = Trehalose at 30 mg ml^{-1}; BABA8000 = DL-3-aminobutyric acid at 8,000 µg ml^{-1}; Some of the treatments includes combinations of BABA (8,000 µg ml^{-1}) and ChitosanA (100 µg ml^{-1}), ChitosanF (1,000 µg ml^{-1}), Bion (100 µg ml^{-1}) PPS (10 µl ml^{-1} or 100 µl ml^{-1}) or Trehalose (30 mg ml^{-1}). PPS10Bion10 or PPS100Bion100 = combinations of PPS at 10 µl ml^{-1} or 100 µl ml^{-1} and Bion at 10 µg ml^{-1} or 100 µg ml^{-1}. The calculations are based upon data from observations of detached leaves from 16 plants within each treatment. The plants were sprayed with the different aqueous solutions 1 week prior to leaf detachment. The detached leaves were incubated at 10°C and light, and inoculated the following day with a spore suspension of *M. majus*

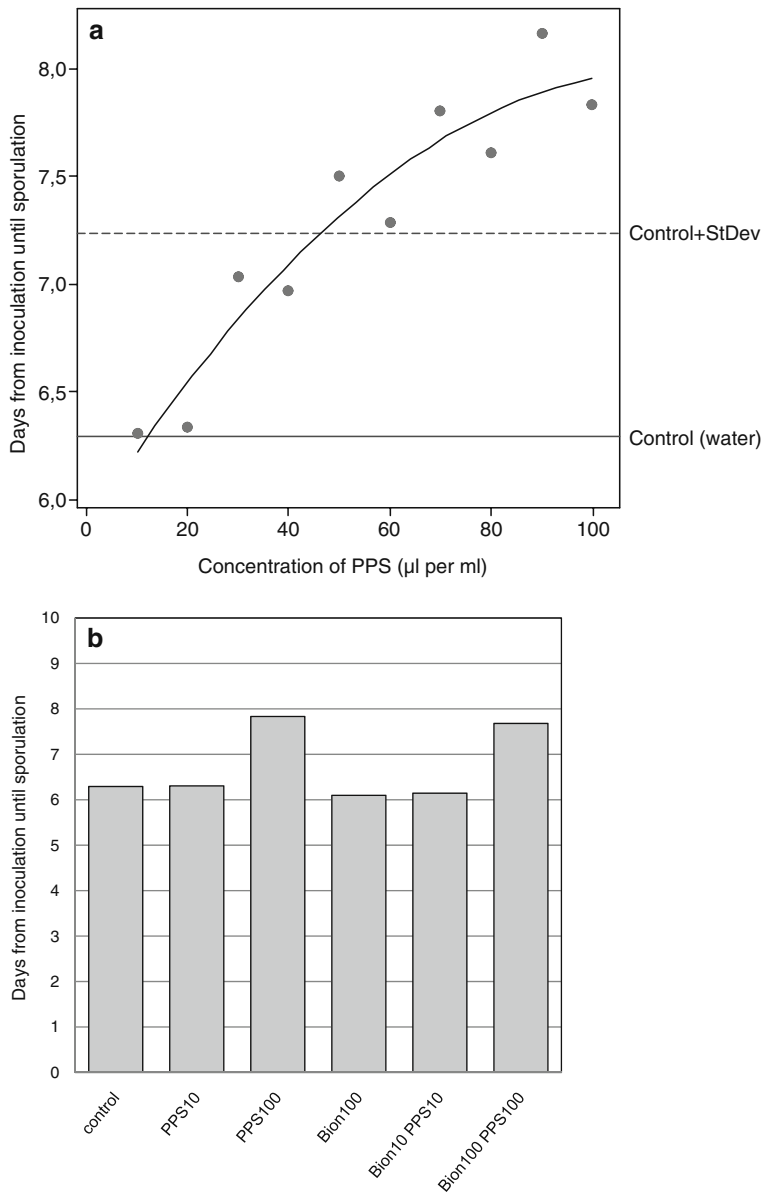

Fig. 3 The effect of potential resistance inducers on sporulation of *Microdochium majus* on detached, inoculated leaves from 3-week old plants of winter wheat (Experiment D). **a** The effect of PPS concentration on days to sporulation presented as curve-of–best-fit by polynomial regression with superimposed horizontal lines for the average value of the control treatment (water treated, inoculated leaves) and control + standard deviation. **b** Days from inoculation until sporulation on leaves pre-treated with PPS, Bion or a combination of the two. * denotes treatment effects significantly different from the water control (GLM in SAS, Dunnett-Hsu, $p<=0.05$). Control = water; PPS10 to PPS100 = a foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution, added in amounts ranging from 10 µg ml^{-1} to 100 µl ml^{-1}; Bion100 = Bion at 100 µg ml^{-1}; PPS10 Bion10 or PPS100 Bion100 = combinations of PPS at 10 µg ml^{-1} or 100 µl ml^{-1} and Bion at 10 µg ml^{-1} or 100 µg ml^{-1}. The plants were sprayed with the different solutions 1 week prior to leaf detachment. The detached leaves were incubated at 10°C and light, and inoculated the following day with a spore suspension of *M. majus*. The figures display average values calculated from tree replicate experiments. Sporulation was registered on 16 detached leaves within treatment in each experiment

combination of Bion and PPS at lower concentrations (PPS (10 µl ml^{-1}) and Bion (10 µg ml^{-1})) or Bion at 100 µg ml^{-1} (Fig. 3b).

FHB, greenhouse studies

Winter wheat was sprayed with solutions containing Bion, BABA or PPS 7 days prior to *F. culmorum* point inoculation of the heads. In the water treated control plants, the average number of bleached spikes per head was 1.2, 3.4 and 8.8 at 11 days, 17 days and 23 days after inoculation, respectively. At 23 days after inoculation, plants pre-treated with PPS at 100 µl ml^{-1} or a combination of PPS (100 µl ml^{-1}) and Bion (100 µg ml^{-1}) displayed a significant reduction (40% or 55%, respectively) in the number of bleached spikelets per spike, compared to water treated, inoculated control (Fig. 4). A significantly reduced disease development was also registered in plants pre-treated with PPS (100 µl ml^{-1}) and Bion

(100 μg ml^{-1}) as compared to control plants at 17 days after *F. culmorum* inoculation, whereas no reduction in symptom development was registered in plants pre-treated with a combination of PPS and Bion at lower concentrations. No reduction in symptom development was registered in plants pre-treated with PPS at 1 μl ml^{-1} and 10 μl ml^{-1} or in Bion- or BABA-treated plants.

In vitro fungal growth

Reduced in vitro growth of *M. majus*, *F. culmorum* and *F. graminearum* was observed when the foliar fertilizer containing potassium phosphite was added into the growth medium. At the lowest PPS concentration used (10 μl ml^{-1}) the mycelial growth of *M. majus* was reduced by more than 90% and no mycelial growth of *M. majus* was registered at PPS concentrations of 50 μl ml^{-1} and 100 μl ml^{-1} (Fig. 5). The mycelial growth of *F. culmorum* and *F. graminearum* was less influenced by PPS. The reduction in mycelial growth rate was about 60–80% at 10 μl PPS ml^{-1}, 80–90% at 50 μl PPS ml^{-1} and more than 90% at 100 μl PPS ml^{-1}.

Discussion

As a pre-screen to select chemical agents for potential FHB disease reduction, development of *M. majus* was studied in detached leaves of winter wheat pre-treated with various putative resistance inducing chemicals. Bion, BABA and a foliar fertilizer containing potassium phosphite were selected to use in the further studies on treatments effects on *F. culmorum* development in heads of winter wheat.

Phosphite is reported to control plant diseases caused by *Phytophthora* spp. in several plant species (Erwin and Ribeiro 1996; Smillie et al. 1989). A fungistatic effect of phosphite was also observed in our experiment both as a reduction of *M. majus* disease development on detached leaves and as a reduced development of *F. culmorum* in heads of winter wheat pre-treated with PPS. Phosphite

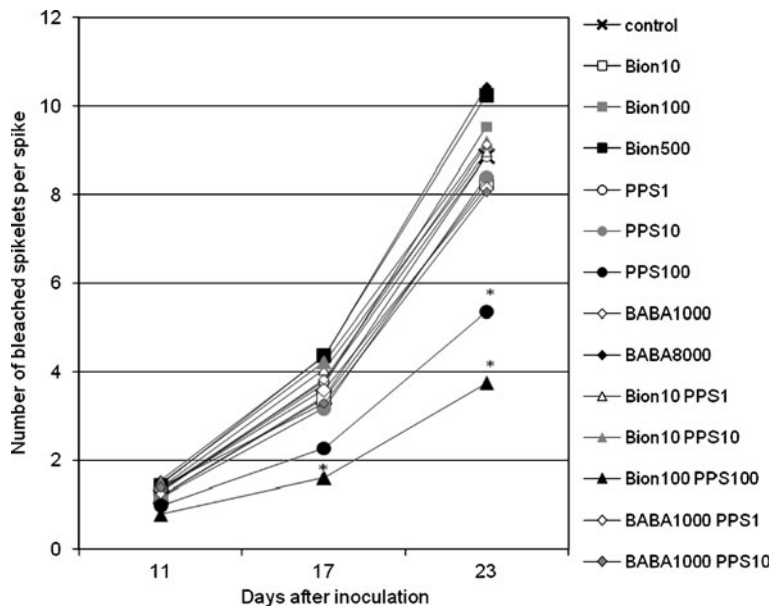

Fig. 4 The effect of potential resistance inducers on development of *Fusarium culmorum* in winter wheat presented as number of bleached spikelets per spike at 11 days, 17 days and 23 days after point inoculation. The plants were sprayed with the following aqueous solutions 1 week prior to point inoculation: control = water; Bion10, Bion100 or Bion500 = Bion at 10 μg ml^{-1}, 100 μg ml^{-1} or 500 μg ml^{-1}, respectively; PPS1, PPS10 or PPS 100 = 1, 10 μl ml^{-1} or 100 μl ml^{-1} respectively, of a foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution; BABA1000 or 8000=DL-3-aminobutyric acid at 1,000 μl ml^{-1} or 8,000 μg ml^{-1} , respectively; Some of the treatments were composed of PPS at a concentration of 1 μl ml^{-1}, 10 μl ml^{-1} or 100 μl ml^{-1} in combination with 10 μg or 100 μg Bion ml^{-1}or 1,000 μg BABA ml^{-1}. Results are presented as mean values from three replicate experiments. *denotes treatment effects significantly different from the water control (GLM in SAS, Dunnett-Hsu, $p<=0.05$).

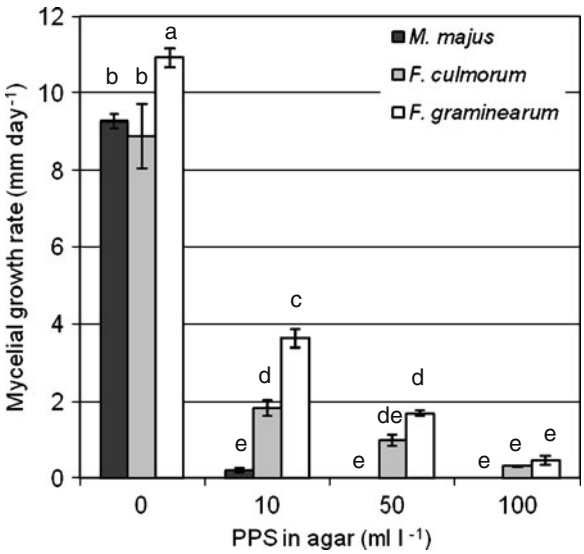

Fig. 5 In vitro mycelial growth rate of *Microdochium majus, Fusarium culmorum* and *Fusarium graminearum* measured on PDA added different amounts of 'PPS' (a foliar fertilizer containing 731 g l^{-1} of a 50% potassium phosphite solution). Mycelial growth rate is expressed as radial growth (mm per day) at 20°C. Standard errors of the mean are indicated and bars with identical letters indicate no significant difference in fungal growth rate between the treatments (GLM in Minitab, Turkey, $p<=0.05$)

remains in the plants for extensive periods after uptake, and there seems to be a close relationship between the concentration of phosphite present at the invasion site and the degree of protection (Smillie et al. 1989). We also observed an increased degree of disease protection in detached wheat leaves from PPS treated plants with increasing concentration of PPS (from 10 ml l^{-1} to 100 ml l^{-1}). In some experiments, we observed necrotic leaf tips on plants sprayed with PPS at concentrations exceeding 60 µl ml^{-1}, while a significant disease reduction of *M. majus* on detached leaves was registered at PPS concentrations from 50 µl ml^{-1}. Thus, a PPS concentration of 50 µl ml^{-1} seems appropriate to avoid phytotoxic effects.

It has been suggested that phosphites slow the growth of the pathogen and inhibit its sporulation and thereby gives the host defence system extra time to react and restrict the growth of the invading pathogen (Erwin and Ribeiro 1996; Smillie et al. 1989). These suggestions are supported by our findings as we observed a reduced in vitro mycelial growth rate of *M. majus*, *F. culmorum* and *F. graminearum* with increasing PPS concentration in addition to a delay in sporulation of *M. majus* on wheat leaves pre-treated with PPS. Our studies could not reveal whether the disease reduction caused by the potassium phosphite solution involves the plants defence system in addition to a direct, fungistatic effect.

A combination of PPS and Bion significantly reduced the disease development of *M. majus* and *F. culmorum* in winter wheat. This was registered as a reduction in *M. majus* sporulation on detached wheat leaves from plants pre-treated with a combination of PPS (100 µl ml^{-1}) and Bion (100 µg ml^{-1}), especially at early time-points after *M. majus* inoculation. A significant reduction of symptom development after *F. culmorum* inoculation was also registered in heads from winter wheat plant pre-treated with a combination of PPS and Bion. Even though no significant effect on disease development was observed in some of the experiments after application of Bion alone, the disease reduction in plants pre-treated with a combination of PPS (100 µl ml^{-1}) and Bion (100 µg ml^{-1}) tended to be stronger than the disease reduction observed from the PPS treatment (100 µl ml^{-1}) alone. Although not significant, shorter incubation period and increased latent period of *M. majus* were observed on detached leaves pre-treated with Bion compared to the water treated controls in some of our experiments. Short incubation period combined with a relatively long latent period was observed in a wheat line ranked as the most FHB resistant line in a study of 30 USA soft winter wheat entries (Browne 2007). A shorter time-period from inoculation until symptom development might therefore not necessarily be a sign of increased susceptibility, but rather indicate an enhanced activation of the plants defence responses. Our observations could therefore indicate that Bion has some influence on the resistance response in the plants we studied. Although BTH (the active ingredient of Bion) is reported to induce resistance towards fungal diseases in wheat (Stadnik and Buchenauer 1999; Görlach et al. 1996), control of FHB has not been achieved by the application of BTH or its analogues (Yu and Muehlbauer 2001; Mohammadi and Kazemi 2002). Studies in wheat have shown that the pathway for induction of defence response genes by *F. graminearum* infection seems to be distinct from the BTH-induced pathway (Yu and Muehlbauer 2001). However, our studies indicate that BTH may have some influence on FHB resistance when

applied in combination with other putative resistance inducers.

BABA seemed to influence disease development in some of our experiments. Although not significant, a shorter incubation period was observed on wheat leaves sprayed with BABA 7 days prior to detachment and subsequent *M. majus* inoculation, compared to water treated leaves in experiment B. Despite no overall reduction in disease severity being observed in heads of winter wheat pre-treated with BABA (1,000 μg ml^{-1}) 7 days prior to *F. culmorum* inoculation, about 10% reduction in FHB disease incidence was registered in BABA-treated plants compared to the water treated control in one replicate of the experiment. β-aminobutyric acid (BABA) is found to induce disease resistance in several plant species (Cohen 2002; Oka and Cohen 2001). The defence responses induced by BABA towards necrotrophic fungi in *Arabidopsis* involve absisic acid and consequent callose deposition, and differs from JA-ISR and SA-ISR (Ton and Mauch-Mani 2004). Resistance towards *F. culmorum* in wheat spike tissue is also associated with rapid depositions of callose in infected tissues (Kang and Buchenauer 2000), which implies that BABA treatment might trigger defence responses of relevance for FHB resistance in wheat.

When the plants were sprayed with a combination of BABA (8,000 μg ml^{-1}) and chitosan F (1,000 μg ml^{-1}) or a combination of BABA (8,000 μg ml^{-1}) and Bion (100 μg ml^{-1}) prior to *M. majus* inoculation, a significant reduction of the incubation period was registered. We also observed small chlorotic/necrotic leaf spots, a possible sign of a plant defence response, in some of the leaves pre-treated with BABA (8,000 μg ml^{-1}) in combinations with other putative resistance inducers. Detached wheat leaves pre-treated with BABA (8,000 μg ml^{-1}) alone or in combination with other potential resistance inducers, displayed an increased latent period (not significant) of *M. majus* in experiment B and a reduced degree of sporulation at early time points after inoculation in experiment C. This was especially noted in leaves pre-treated with a combination of BABA (8,000 μg ml^{-1}) and Chitosan A (100 μg ml^{-1}). Enhanced disease reduction was registered in *Fusarium sambucinum* inoculated potato pre-treated with a combination of chitosan and BABA, compared to chitosan treatment alone (Greyerbiehl and Hammerschmidt 1998). Although not consistent, our studies indicate that BABA, especially in combination with other putative resistance inducers, has some influence on the disease resistance response of winter wheat.

Pre-treatment of wheat with chitosan alone did not reduce development of *M. majus* in detached leaves in our study. The formulations, timing of application, chitosan concentrations or the method we used might not be suitable for this purpose. In combination with other putative resistance inducers, chitosan tended to have some influence on disease development of *M. majus* in our study. Chitosan treatment of wheat is reported to reduce wheat coleoptile growth retardation and seedling blight caused by *F. culmorum* (Khan et al. 2006). In addition to a longer latent period, we observed small chlorotic/necrotic leaf spots in some of the leaves pre-treated with chitosan alone or in combinations with BABA. Preparations of chitin or chitosan have been reported to trigger defence reactions and thereby potentially induce disease resistance in wheat (Vander et al. 1998; Kuwabara et al. 2002; Barber et al. 1989; Hofgaard et al. 2005). Our and previous results indicates that there might be possibilities for control of *Fusarium* spp. in wheat by a combination of chitosan and other putative resistance inducers.

Although there was a tendency towards a reduction in disease development of *M. majus* on detached leaves pre-treated with several of the known resistance inducers used in our study, the effect was not consistent. Detaching the wheat leaves prior to *M. majus* inoculation could have interfered with the effect of the different treatments in our study. Wheatwin1, a pathogenesis-related protein of class 4 (PR4) from wheat, has antifungal activity against *F. cumlorum* (Caporale et al. 2004). Induction of genes encoding wheatwin1 has been observed in wheat in response to wounding, *F. culmorum* infection and after treatments with resistance inducers (Bertini et al. 2003). Thus, wounding might trigger some of the same defence responses as the resistance inducer treatments. Other studies indicate that the effect of a resistance inducer might not be fully expressed in detached leaves. The concentration of gramine, a proposed marker for ISR in barley, did not increase in detached primary leaves but a significant increase in gramine was measured in the parts of these leaves that still remained on the seedling (Matsuo et al. 2001). However, induced resistance to powdery mildew has successfully been observed in detached wheat leaves

after treatment with putative defence activators (Reignault et al. 2001; Stadnik and Buchenauer 1999). The fact that the effect of the potassium phosphite solution was more expressed in our study of whole plants, compared to in experiments using detached leaves, could indicate that a potential resistance-inducing effect was masked in the detached leaf assay. This might be due to a resistance activating effect of the detachment itself or by a reduced ability of the detached leaves to respond upon inoculation, or both.

The effect of putative resistance inducers in plants can also be influenced by factors such as concentration, timing, leaf stage and light regime after inoculation (Newton and Dashwood 1998). The relatively long time-period (1 week) between application of the different putative resistance inducers and inoculation in our study can have influenced or reduced the effect of the different treatments. This period was chosen because we were aiming for treatments of relevance for FHB reduction in practise. Enhanced resistance towards powdery mildew has been registered in wheat with increased time period between BTH treatment and inoculation from 1 day to 4 days, and a clear disease reduction was also observed in plants pre-treated 7 days prior to inoculation (Görlach et al. 1996). Although resistance has been induced in other plant-pathogen systems despite a long time period between treatment and inoculation, the optimal timing might differ between treatments in combination with the host plant-pathogen system.

By using detached leaves from 3-week old seedlings instead of mature wheat plants to screen for putative resistance inducers for FHB control, the time spent on screening for effective treatments were markedly reduced. A problem by using this detached leaves assay could be that chemicals effective in controlling *M. majus* does not always display the same efficacy in controlling *Fusarium spp*. Still, *M. majus* is often isolated from wheat heads displaying FHB symptoms and it might therefore be advantageous if treatments aimed for control of *Fusarium* spp. also displayed a significant control of *M. majus*. The treatment (PPS) we found most efficient in controlling *F. culmorum* in heads of winter wheat was first selected due to its ability to reduce development of *M. majus* on detached leaves. This implies that a detached leaf assay using *M. majus* can be used as a screening method to select treatments for potential FHB control.

We conclude that development of *M. majus* and *F. culmorum* in winter wheat can be reduced by application of high concentrations of a foliar fertilizer containing potassium phosphite. Our studies could not reveal whether the disease reduction caused by this treatment involves the plants defence system in addition to a direct (fungistatic) effect. The effect of potassium phosphite on development of FHB should be further studied.

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