

Effect of an NTPDase Inhibitor on *Leptosphaeria Biglobosa* in *Brassica Napus*

Songbai Rong, Qiangsheng Li, Fengxiang Chen, Xinjie Wu and Mingguang Chu*

Anhui Academy of Agricultural Sciences, China

ABSTRACT

Leptosphaeria biglobosa, which causes blackleg on canola and vegetable crucifers, is an important pathogen in China. Germination of fungal spores and hyphal growth play a key role in fungal infection and development of the disease. The current study examined the effect of the NTPDase inhibitor chlorpromazine (CPZ) on morphological changes in the fungus *Leptosphaeria biglobosa*. The conidial germination, rate of hyphal growth and pathogenicity of the blackleg were investigated after pretreatment of the fungus with different concentrations of CPZ. The study demonstrated that various aspect changes of the fungus were significantly affected by the CPZ, indicating NTPDase is essential in the infection of *Brassica napus* cultivars.

KEYWORDS: Nucleoside Triphosphate Diphosphohydrolase; Inhibitor; *Leptosphaeria biglobosa*; *Brassica napus*

INTRODUCTION

Blackleg is an important disease of cruciferous crops, especially Brassica species. It causes leaf lesions on the autumn and winter followed by a stem canker in early summer on winter canola in the oilseed production areas of the world [1-3]. Air-borne ascospores released from the infected residues of the winter-type oilseed rapes are the air-borne primary inoculum for the initiation of the disease [4,5]. The germinated ascospores invade leaves to form lesions, then the fungus grows down petioles into the stems to infect the upper or basal stems to produce typical symptoms [6]. Stem can also be directly infected by the splashed pycnidiospores from the leaf and cotyledon lesions [7]. Two pathogens, *Leptosphaeria maculans* and *L. biglobosa*, were identified in blackleg based on their differences in cultural, molecular and biochemical characteristics [5,8-10]. Outbreaks of *L. biglobosa* is becoming prevalent in different geographical locations and has reduced oilseed rape yield by 9.1-33.1% in different years in China [11,12], but *L. maculans* is not yet a problem.

Nucleoside triphosphate diphosphohydrolases (NTPDases) are a large class of evolutionarily conserved enzymes that are present in many eukaryotic organisms and play important roles, serving as nucleotidases that hydrolase NTPs and NDPs to monophosphate

[13,14]. The importance of NTPDases has been investigated in a number of organisms including human, yeast, and medical pathogens [15-17], however, the function of NTPDases in the fungus *L. biglobosa* remains largely unknown. The current report showed that the role of NTPDase inhibitor CPZ was related to the infection of oilseed rape. The findings from this report may provide a new method to study the pathogenicity of *L. biglobosa* and to control the disease.

MATERIALS AND METHODS

Brassica napus cultivars: The winter-type oilseed rape cultivar Zhongheza 418 and the spring-type cultivar Westar, were used in this experiment.

Isolation and sporulation: In early summer, 2015, isolation of *L. biglobosa* was conducted from the fresh plants grown in the experimental fields or plant residues in Anhui, China. The procedure was carried out as follows: after rinsed with tap water to remove the soil on the surface of the material, the items were cut into small pieces. The tissue with typical blackleg symptoms was placed into 75% ethanol for 20 seconds and transferred into 1% sodium hypochlorite for 3 minutes to surface sterilize, then it was

Quick Response Code:



Address for correspondence: Mingguang Chu, Crops Institute, Anhui Academy of Agricultural Sciences, China

Received: January 22, 2021

Published: January 27, 2021

How to cite this article: Songbai R, Qiangsheng L, Fengxiang C, Xinjie W, Mingguang C. Effect of an NTPDase Inhibitor on *Leptosphaeria Biglobosa* in *Brassica Napus*. 2021- 3(1) OAJBS.ID.000249. DOI: 10.38125/OAJBS.000249

washed in sterile water for 20 minutes. The water on the surface of the items was absorbed with sterile paper. Finally, the pieces were placed on water agar medium in a Petri dish (the diameter 19cm) and incubated at 22 °C for 2 days.

When the colony extended across the water agar medium, the tips of the hypha were cut and transferred onto the potato dextrose agar (PDA) and incubated at 22 °C. To purify the pathogen, the hyphal tips of the growing colony were removed and transferred onto new PDA media after 3 days of incubation at 22 °C. The purification of the pathogen on the PDA medium was repeated 3 times. The strain was subcultured on the V-8 juice agar for sporulation. Some pure cultures of the strain were maintained on PDA at 4 °C for future inoculation.

Preparation of spore suspension: A total of 10 sporulating cultures on the V-8 juice agar were flooded with sterile distilled water and scraped gently with a bent glass rod to dislodge the conidial spores. The pooled pycnidiospore mixture was filtered through eight layers of gauze and centrifuged at 2000rpm for 5min. The supernatant was transferred into a new tube, and the concentration of spore suspension was adjusted to 1×10^7 spore ml⁻¹ using a hemocytometer and stored at -20 °C in the freezer until required.

Treatment of the spores with the CPZ: NTPDase inhibitor chlorpromazine (CPZ) was dissolved in ddH₂O and diluted to 100mM. Aliquots of 0.5ml spore suspension was transferred into new tubes and added to the inhibitor solution to get final concentrations of 20μM, 40μM and 60μM of CPZ, respectively, which were incubated at 22 °C for 3h. Spores treated with ddH₂O were included as control.

Observation of conidial germination: The CPZ on the surface of spores was removed by centrifugation at 5,000rpm for 3min and was washed three times with ddH₂O, then resuspended in ddH₂O and incubated at 22 °C for 3h. After incubation, 20μl of the spore solution was loaded onto the glass microscope slides to calculate the germination rates with a microscopy. A total of 200 spores were examined in each replicate, and 3 replicates were carried out. The experimental designs were conducted in 2015, 2016 and 2017, respectively.

Measurement of hyphal growth speed: Plugs (diameter 2mm) were prepared by cutting the margin of actively growing colonies of the isolates and placing them in Petri dishes containing CPZ with the concentration of 20μM, 40μM and 60μM. After 3h of incubation

at 22 °C, the plugs were washed 3 times with ddH₂O to remove CPZ from the surface of the inoculum. 50 treated plugs were transferred onto the PDA medium for observation of hyphal growth. After incubation for 4 to 10 days at 22 °C, the diameters of colonies on PDA were measured. The experimental design was repeated three times.

The test of pathogenicity: The pathogenicity of the isolates on the rapeseed was carried out in a growth chamber. Seeds were grown in 50-well trays containing peat moss in a growth chamber at 22 °C with a photoperiod of 16h light and 8h dark. After 10 days of sowing, growing tips of the seedlings were removed every 2 or 3 days to ensure the cotyledons remained green.

The previously described pretreated plugs were used to inoculate the cotyledons. Seedlings were wounded with a needle at the centre of the cotyledon and inoculated with a treated plug by placing it over the wound. PDA and plugs without CPZ were used as controls. The treated seedlings were kept in a dew chamber set at 22 °C for 24h before being moved to growth chamber for disease development. A randomized complete block design was used, with 3 replicates (50 plants / tray) per treatment. The experiment was carried out 3 times.

Severity of blackleg symptoms on individual plants was rated as follows: Treated cotyledons were assessed by scoring the symptoms on a scale of 0-9 after 12 days of inoculation using techniques based on the extent of the necrosis at the cotyledon described by Koch [18] and Li [19]. A mean blackleg index in each replicate was calculated from the formula:

$$\text{Disease index (DI)} = \frac{\sum (n \times 0 + n \times 1 + n \times 2 + \dots + n \times 9)}{N}$$

Where N is the total number of plants, n is the number of plants in each class and 0, 1, 2, … and 9 are the symptom severity classes.

RESULTS

Suppression of the hyphal growth by the treatment with CPZ: CPZ showed inhibitory effects on hyphal growth. After a period of incubation at 22 °C, hypha which were pretreated with CPZ exhibited morphological changes. The rate of growth of the colonies on the PAD in the presence of CPZ was slower compared to that of the controls, and decreased more as presence inhibitor concentration rose at early stage (before 7th day), but the inhibitory effect of higher CPZ concentration (40μM and 60μM) declined by the 10th day (Figure 1).

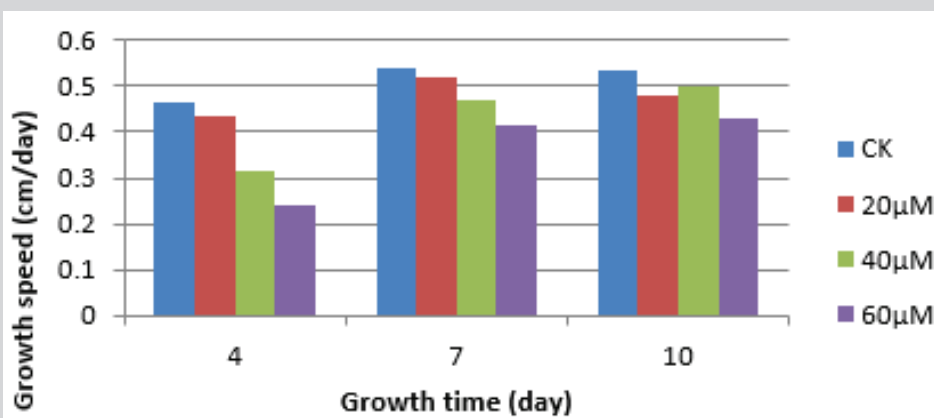


Figure 1: CPZ suppression on *L. biglobosa* hyphal growth

Conidial germination rate: The germination of conidia pretreated with CPZ declined by 18.8% at the lower inhibitor concentration (20 μ M) and dropped 81.2% at the higher inhibitor concentration (60 μ M) compared to that of the controls (Figure 2). At the same time, it was observed that germ tube length was shorter with the highest inhibitor concentration.

Pathogenicity tests: Cotyledons inoculated with pretreated plugs with CPZ showed different symptoms compared to these

of the control. The average disease index (DI) decreased with the treatment of CPZ (Figure 3).

At 7 days r inoculation, there were significant ($p < 0.01$) differences in the mean size of the lesions on the cotyledons between the treatments and the control, the DI of the seedlings inoculated with pretreated plugs was 1.4, 1.6 and 1.6 for Westar, and 2.3, 1.1 and 1.1 for ZHZ418, respectively.

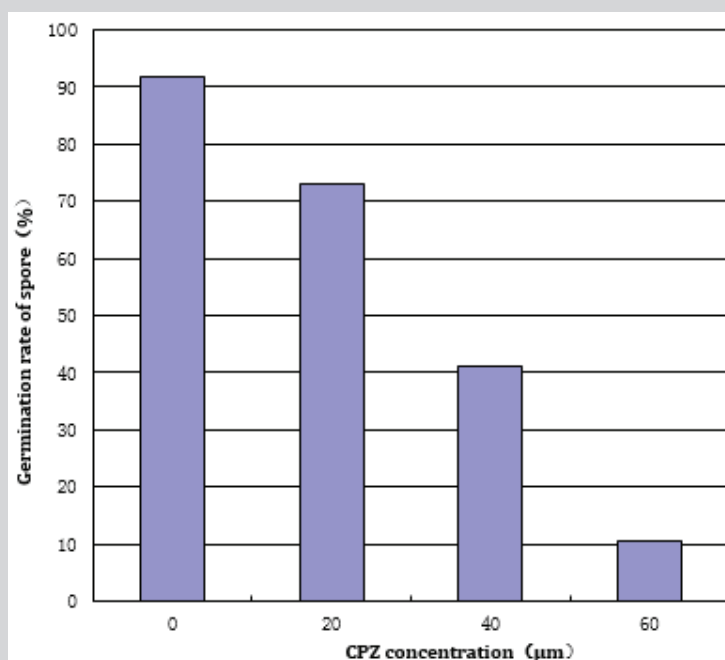


Figure 2: Conidial germination with CPZ different concentration.

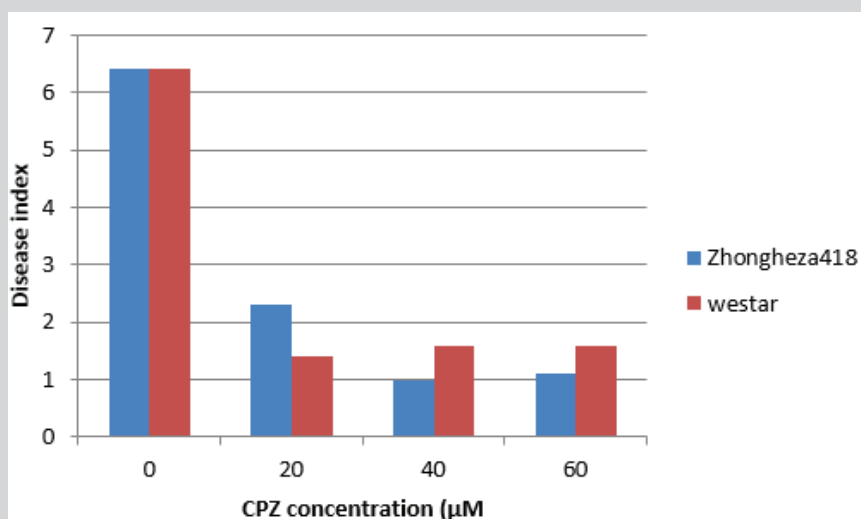


Figure 3: CPZ reduced pathogenicity of *L. biglobosa*.

DISCUSSION

In previous studies, spore suspensions were often used for cotyledon inoculation to assess the pathogenicity of blackleg [20-24]. It was effective for the study of blackleg fungus under various conditions, but the droplets of the spore suspension were easy to displace from the wound or fall down from the cotyledon even if the adhesive agent Tween 40 was added in the solution if the seedlings

were moved during the experiment. In this study, agar plugs were utilized as the inoculum instead of spore suspension, and the measures of the hyphal plug and spore suspension inoculation on the cotyledon were both useful. We developed a new method for the blackleg inoculation in *Brassica napus*.

Long et al. [25] demonstrated that the NTPDase inhibitors CPZ, TDZ and TPZ could reduce the conidial germination and appressoria formation of the rice blast fungus *Magnaporthe oryzae*. In the

current study, the NTPDase inhibitor CPZ decreased the mycelial growth, spore germination of pathogen *L. biglobosa* and lesion area on the cotyledons of oilseed rape. The DI showed different decline on Zhongheza 418 and Westar during the inoculation, maybe have the relation with their various background of the cultivars. The next step would be to check the effects of inhibitors TPZ and TDZ on the causal agent *L. biglobosa* in different cultivars on *Brassica napus*.

Li et al. [26] reported that ascospore germination, penetration, and development of symptoms on cotyledons of spring-type canola were much earlier than that with pycnidiospores. In the current study, the pycnidiospore was utilized as inoculum. Since isolates from various geographical environments have differential pathogenicity to cultivated rape, single monospore cultures of different isolates of *L. biglobosa* should be tested.

ACKNOWLEDGEMENT

We thank professor Sheau-Fang Hwang for the revising of the manuscript, and professor Chen Fengxiang and Professor Wu Xinjie for their technical assistance on the experiment. This work was supported by National Key Research and Development Program of China (2018YFE0108000-4, 2018) and the Independent Innovation Project of Anhui Academy of Sciences (17A0206).

REFERENCES

- McGee DC, Petrie GA (1978) Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. *Phytopathology* 68(4): 625-630.
- Khangura RK, Barbetti MJ (2001) Prevalence of blackleg (*Leptosphaeria maculans*) on canola (*Brassica napus*) in western Australia. *Animal Production Science* 41(1): 71-80.
- Larkan NJ, Yu F, Lydiate DJ, Rimmer SR, Borhan MH (2016) Single R gene introgression lines for accurate dissection of the *Brassica*-*Leptosphaeria* pathosystem. *Front. Plant Sci* 7: 1771.
- Gladders P, Musa TM (1980) Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. *Plant Pathology* 29(1): 28-37.
- West JS, Kharbanda PD, Barbetti MJ, Fitt BDL (2001) Epidemiology and management of *Leptosphaeria maculans* (Phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathol* 50(1): 10-27.
- Hammond KE, Lewis BG, Musa TM (1985) A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. *Plant Pathol* 34: 557-565.
- Boker A, Barbetti MJ, Brown AGP, MacNish GC, Wood PMcR (1975) Blackleg of rapeseed. *Journal of Agriculture of Western Australia* 16: 7-10.
- Williams PH (1992) Biology of *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* 14: 30-35.
- Williams RH, Fitt BDL (1999) Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape in UK. *Plant Pathol* 48(2): 161-175.
- Fitt BDL, Brun H, Barbetti MJ, Rimmer SR (2006) World-wide importance of Phoma stem canker (*Leptosphaeria maculans* and *L. biglobosa*) on oilseed rape (*Brassica napus*). *European Journal of Plant Pathol* 114: 3-15.
- Li QS, Hu BC, West JS, Chen FX, Fei WX, et al. (2008) Phoma stem canker on oilseed rape. *Edible Fungi of China* 27(suppl): 90-93.
- Rong SB, Hu BC, Chen FX, Wu XJ, Hou SM, et al. (2015) Effect of *Leptosphaeria biglobosa* on yields and agricultural treats of canola in China. *Crops* 6: 159-161.
- Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *BBA Mol Cell Res* 1783(5): 673-694.
- Zimmermann H, Zebisch M, Sträter N (2012) Cellular function and molecular structure of ecto-nucleotidases. *Purinergic Signal* 8(3): 437-502.
- Knowles AF (2011) The GDA1_CD39 superfamily: NTPDases with diverse functions. *Purinergic Signal* 7: 21-45.
- Sansom FM (2012) The role of the NTPDase enzyme family in parasites: what do we know and where to from here? *Parasitology* 139: 963-980.
- Zebisch M, Schäfer P, Lauble P, Sträter N (2013) New crystal forms of NTPDase1 from the bacterium *Legionella pneumophila*. *Acta Cryst F* 69: 257-262.
- Koch E, Song K, Osborn TC, Williams PH (1991) Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. *Mol Plant-Microbe Interact* 4(4): 341-349.
- Li QS, Rong SB, Hu BC, Jiang YF, Hou SM, et al. (2013) Distribution of blackleg disease on oilseed rape in China and its pathogen identification. *Chinese J of Oil Crop Sci* 35(4): 415-423.
- Mahuku GS, Goodwin PH, Hall R, Hsiang T (1997) Variability in the highly virulent type of *Leptosphaeria maculans* within and between oilseed rape fields. *Can J Bot* 75: 1485-1492.
- Yu F, Lydiate DJ, Rimmer SR (2005) Identification of two novel genes for blackleg resistance in *Brassica napus*. *Theor Appl Genet* 110: 969-979.
- Liu SY, Liu Z, Fitt BDL, Evans N, Foster SJ, et al. (2006) Resistance to *Leptosphaeria maculans* (Phoma stem canker) in *Brassica napus* (oilseed rape) induced by *L. biglobosa* and chemical defence activators in field and controlled environments. *Plant Pathol* 55: 40-412.
- Long Y, Wang Z, Sun Z, Fernando WGD, McVetty BEP et al. (2011) Identification of two blackleg resistance genes and fine mapping of one of these two genes in a *Brassica napus* canola variety 'surpass 400'. *Theor Appl Genet* 122: 1223-1231.
- Haddadi P, Larkan NJ, Borhan MH (2019) Dissecting R gene and host genetic background effect on the *Brassica napus* defense response to *Leptosphaeria maculans*. *Scientific Reports* 9: 6947.
- Long B, Li GH, Feng RR, Tao L, Liu DD, et al. (2015) NTPDase specific inhibitors suppress rice infection by magnaporthe oryzae. *Journal of Phytopathology* 163(3): 227-232.
- Li H, Sivasithamparam K, Barbetti MJ, Kuo J (2004) Germination and invasion by ascospores and pycnidiospores of *Leptosphaeria maculans* on spring-type *Brassica napus* canola varieties with varying susceptibility to blackleg. *J Gen Plant Pathol* 70: 261-269.