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# Effects of fungicides JS399-19, azoxystrobin, tebuconazloe, and carbendazim on the physiological and biochemical indices and grain yield of winter wheat

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ABSTRACT

The impact of four modern fungicides JS399-19 (2-cyano-3-amino-3-phenylancryic acetate) (novel fungicide), azoxystrobin (a strobilurin), tebuconazole (a triazole) and carbendazim (a benzimidazole), applied as foliar spray at the recommended field rate, on the physiology and biochemistry of the senescence process and grain yield was studied in winter wheat (*Triticum aestivum* L. cv. 'Nannong No. 9918') under natural environmental conditions. Fungicide treatments to wheat plants at growth stage [ZGS] 57 (3/4 of head emerged) significantly increased the chlorophyll (CHL) and soluble protein (SP) content and decreased the malondialdehyde (MDA) content and electrolyte leakage. Additionally, activities of the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in flag leaves of the fungicide-treated plants were also higher than that in untreated plants. These coincided with elevated levels of $H_2O_2$ and reduced level of $O_2^-$ in the fungicide-treated plants. The results suggested that the fungicide-induced delay of senescence was due to an enhanced antioxidant enzyme activity protecting the plants from harmful active oxygen species (AOS). Because all fungicides can induce the delay of wheat senescence, fungicide-treated wheat shown higher grain yield than untreated wheat. Of all tested fungicides, JS399-19, azoxystrobin and tebuconazole showed similar effects on delaying senescence of wheat and enhancing the grain yield of wheat, but JS399-19 was more efficient in general.



## 1. Introduction

As a staple food for more than 35% of the world population, wheat (*Triticum aestivum* L.) is the second grain crop in China and its production status is directly related to social stability, Chinese survival and sustainable development [1–3]. In China, the cultivation area for wheat has reached $3.12 \times 10^5$ ha$^2$ and the total wheat production has been more than $1.04 \times 10^6$ t, which is the biggest country for wheat production and consumption in the whole world [3]. As we all know, the grain filling relied on the duration of photosynthetically active leaf area and the last function of flag leaf [4]. Therefore, natural and unnatural senescence of flag leaf will affect the wheat production. In addition, the reduction of wheat yield also can be caused severely by fungal diseases from the flowering to the harvest.

Leaf senescence is an oxidative process that generates active oxygen species (AOS) such as hydroxyl ($OH^{\cdot}$) and superoxide radicals ($O_2^-$), singlet oxygen ($^1O_2$), and hydrogen peroxide ($H_2O_2$) [5]. Active oxygen species in the aging process involves various biochemical and physiological changes during leaf senescence, such as pigment degradation, alteration of membrane permeability, breakdown of proteins, altered phytohormone metabolism, and nucleic acids degradation [6,7]. These changes result in dramatically reduced growth and productivity, abnormal development, and eventual death of some plants [7]. To prevent such damage, plants have evolved various protective mechanisms to eliminate or reduce AOS. One of the protective mechanisms is the enzymatic antioxidant system including superoxide dismutases [EC 1.15.1.1] (SOD), catalases [EC 1.11.1.6] (CAT), and peroxidases [EC 1.11.1.7] (POD) [8]. SOD dismutated $O_2^-$ to $H_2O_2$, and $H_2O_2$ was metabolized into $H_2O$ by CAT and POD.

Over the years great progress in theory about plant senescence had been achieved. We have known that AOS were closely related to plant senescence, because of AOS act as crucial signals in regulation of plant senescence [9]. In addition, much research has partially revealed reaction pattern between AOS and abiotic or biotic stress, such as salt stress, cold stress, high temperature stress, acid stress, alkaline stress, pathological reactions, senescence, growth, development, cell cycle, UV-B damage, wounding, embryogenesis and flowering [10–15]. But less attention has been addressed towards the effect of fungicide on plant senescence. And most of these investigations mostly focus on the effectiveness of fungicides in reducing AOS and inducing antioxidant system to prevent plants from harmful abiotic or biotic factors in greenhouse condition

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[4,6,16–18]. In other words relatively little work has focused on the physiological and biochemical changes of plants treated with fungicides alone under field condition.

The chemicals of strobilurins, triazoles and benzimidazoles were very important fungicides with different mode of action and they have been shown to have antioxidative properties [6,19]. JS399-19, 2-cyano-3-amino-3-phenylancryic acetate, is a new class of systemic fungicide with a unique mode of action introduced to control Fusarium head blight (FHB) of wheat in China [20,21] and its effect on the physiology and biochemistry of the wheat senescence process has not been studied. In the present study, we investigated the effect of novel fungicide JS399-19, strobilurin azoxystrobin, triazole tebuconazole and benzimidazole carbendazim, on the physiology and biochemistry of the senescence process and grain yield of winter wheat, with specific emphasis on the involvement of the oxidative/antioxidative status of the plants.

## 2. Materials and methods

### 2.1. Plant material and fungicide application

During the growing season of 2006, winter wheat (*T. aestivum* L. cv. 'Nannong No. 9918') was grown according to normal agronomic practice on Jiangpu Farm (Experiment Farm of Nanjing Agricultural University in China). To avoid side-effect of herbicide, we removed weed mechanically. When plants were at Zadoks growth stage [ZGS] 57 (3/4 of head emerged) [22], uniform wheat were chosen and divided into 30 plots (each plot 2.5 m × 4.0 m). Plots were arranged according to a randomized block design with six replicates for each treatment. At [ZGS] 59 (ear emergence), four fungicides plus water control were sprayed on the foliage in the plot from both sides until runoff. The following fungicide dose was used: carbendazim 375 g a.i. $Ha^{-1}$; tebuconazole 125 g a.i. $Ha^{-1}$; azoxystrobin 250 g a.i. $Ha^{-1}$; JS399-19 375 g a.i. $Ha^{-1}$. The experiment was repeated in the same field in the growing season of 2007.

### 2.2. Plant sampling, FHB disease assessment and yield determination

At [ZGS] 60 (Beginning of flowering), [ZGS] 65 (Flowering half complete), [ZGS] 73 (early milk), [ZGS] 80 (beginning dough) or [ZGS] 87 (hard dough), 50 flag leaves of wheat were arbitrarily taken from each plot per treatment. One part of leaves was frozen in liquid $N_2$ and stored at −85 °C until extraction for enzyme analyses. The other part of leaves was used for chlorophyll, soluble protein, lipid peroxidation, superoxide radicals ($O_2^-$), and hydrogen peroxide ($H_2O_2$) assays immediately. The FHB disease was assessed by counting the number of infected spikelets per head 21 days after fungicides application and expressed as the incidence of infected spikelets. Fifty heads were arbitrarily chosen and analyzed for each plot per treatment. In addition, a homogeneous 500 g sample of grain at 13% moisture was taken from each plot per treatment at [ZGS] 92. A 100 g sub-sample of grain was arbitrarily selected to determine the shriveled grain number per 1000 grains, grain number per spike, and 1000 grain weight.

### 2.3. Measurement of chlorophyll (CHL) and soluble protein (SP)

The CHL content in wheat (*T. aestivum*) was determined in 80% acetone extract of 0.2 g leaf as described by Hegedüs et al. [23] and expressed as mg $g^{-1}$ fresh weight (FW).[2] Soluble proteins were assayed by the method of Bradford [24] using bovine serum albumin (BSA) as standard and expressed as mg $g^{-1}$ FW. The absorbance of sample was measured using a spectrophotometer (Beckman, CA, USA).

### 2.4. Measurement of lipid peroxidation and electrolyte leakage

As a biomarker of lipid peroxidation, malondialdehyde (MDA) was measured by TBA method [25] and expressed as μmol $g^{-1}$ FW. Leaf electrolyte leakage was assayed as relative conductivity following the method [26] and calculated according to the formula (first conductivity/second conductivity) × 100. Conductivity was measured using a conductivity meter (Eutech Instruments Pte. Ltd., Ayer Rajah Crescent, Singapore).

### 2.5. Measurement of active oxygen species (AOS)

The superoxide radicals ($O_2^-$) assay followed the method [27] modified from Wang and Lou [28]. The procedures and conditions of the assay were as follows: fresh leaf samples (0.5 g) from control and treated plants were ground with 5 ml homogenization buffer containing 195 μM phosphate buffer (pH 7.8), 10 μM hydroxylammonium chloride and 100 μM EDTA-$Na_2$ in ice bath. The homogenates were centrifuged at 13,000*g* for 30 min at 4 °C with a centrifuge (Beckman, CA, USA). One milliliter supernatants were transferred into fresh tubes, adding 1 ml of 17 mM sulphanilamide (in 30% acetic acid) and 1 ml of 7 mM naphthalene diamine dihydrochloride into each tube in order and incubated for 10 min at 37 °C. Then add 3 ml ether into each tube, fully blended and centrifuged at 5000*g* for 5 min at room temperature. The absorbance of the lower aqueous phase was measured at 540 nm by a spectrophotometer (Beckman, CA, USA). Calibration curves were established from 0 to 250 μM $NO_2^-$. The $r^2$ between $OD_{540}$ and $[NO_2^-]$ was 0.9996. From the following reaction: $2O_2^- + H^+ + NH_2OH \rightarrow H_2O_2 + H_2O + NO_2^-$ the concentration of $O_2^-$ was calculated according to $[O_2^-] = 2[NO_2^-]$ (μM) from the calibration curve.

The hydrogen peroxide ($H_2O_2$) assay followed the method of Lin et al. [29]. Fresh leaf samples (0.5 g) were ground with acetone in ice bath and the volume was up to 5 ml finally. The homogenates were centrifuged at 5000*g* for 5 min at room temperature. One milliliter supernatants were transferred into 2 ml Eppendorf tubes, adding 100 μl 20% $TiCl_4$ solution (dissolved in 37% hydrochloric acid) and 200 μl of ammonia into each tube. The mixtures were centrifuged at 12,000*g* for 10 min at room temperature. The resultant precipitates were washed thrice and re-dissolved in 3 ml 1 mol $L^{-1}$ vitriol. The absorbance was measured at 410 nm by a spectrophotometer (Beckman, CA, USA). Calibration curves were established from 0 to 10 mM $H_2O_2$. The $r^2$ between $OD_{410}$ and $[H_2O_2]$ was 0.9971. The concentration of $H_2O_2$ was calculated from the calibration curve.

### 2.6. Measurement of antioxidative enzyme activity

For enzyme extracts, fresh leaf samples (1 g) from control and treated plants were ground with 5 ml pre-cooled 50 mM Na–phosphate buffer (pH 7.8), 0.1 mM EDTA-$Na_2$ and 1% (w/w) PVP. The homogenates were centrifuged at 13,000*g* for 30 min at 4 °C and the resulting supernatants were used for enzyme assay. All enzyme activity data were related to plant fresh weight (FW).

Superoxide dismutase (SOD) activity was estimated by recording the decrease in optical density of nitroblue tetrazolium (NBT) dye by the enzyme [30].Three milliliter of the reaction mixture contained 0.3 ml 13 mM methionine, 0.3 ml 75 μM NBT, 0.3 ml 0.1 mM EDTA-$Na_2$, 1.5 ml 50 mM phosphate buffer (pH 7.8), 0.5 ml distilled water and 0.1 ml crude extract. Reaction was started by adding 0.3 ml 2 μM riboflavine and placing the tubes

[2] *Abbreviations used:* FW, fresh weight; CHL, chlorophyll; SP, soluble protein; MDA, malondialdehyde; $O_2^-$, superoxide radicals; $H_2O_2$, hydrogen peroxide; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase.