Title

Potassium influenced phenylalanine ammonia-lyase, peroxidases and polyphenol oxidases in Fusarium graminearum infected maize (Zea mays L.)

Authors

Li, Wenjuan
He, Ping
Jin, Jiyun

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Introduction

Stalk/root rot caused by several species of pathogens, such as *Fusarium graminearum* and *Diplodia* (Stack 1999) is a serious and widespread disease in maize (*Zea mays L.*) reducing both yield and quality (Sobek and Munkvold 1999). The disease is commonly found to be more destructive to the crop when grown on potassium deficient soil (Ahmad et al. 1996). Potassium (K) is an essential plant nutrient influencing crop metabolism, growth, development, and yield. Potassium has been shown to promote plant disease reduction and potassium stress can increase the degree of crop damage by bacterial and fungal diseases (Kettlewell et al. 2000, Holzmueller et al. 2007, Grewal and Williams 2002). However, the mechanism by which potassium stimulates resistance towards pathogens is not completely understood (Amtmann et al. 2008). Increases in the activities of enzymes involved in the metabolism of phenolic compounds have been correlated with resistance of cereals to biotic stresses (Mohammad and Kazemi 2002). Phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), Peroxidase (POD; EC 1.11.1.7), Polyphenol oxidase (PPO; EC 1.10.3.2 or EC 1.14.18.1) induction has been linked to defence responses that are involved in resistance towards numerous diseases (Tovar et al. 2002; Quiroga et al. 2000; Mayer 2006). K can enhance the resistance of maize to stalk rot, and the activities of defense-related enzymes (PAL, POD, PPO) are good indicators for resistance to biotic stress (Lo et al. 1999).

The objective of this study was to investigate potential mechanisms by which potassium regulates stalk rot resistance. We focused on the activities of PAL, POD and PPO, and the expression of their corresponding genes, in potassium-treated and non-treated maize roots, which were inoculated with *Fusarium graminearum*.

Materials and Methods

Cultivation of plants

Maize (*Zea mays* L.) seeds of the variety Ji Dan 180 were surface sterilized with H_2O_2 (10%) for 20 min, rinsed thoroughly with distilled water, and germinated on moist filter paper in an incubator at 25 °C. Three days later, uniformly germinated seeds were selected and sown in plastic pots (three seeds to a pot) filled with 450 g quartz sand. The plants were grown in a glasshouse and watered with 1/3 strength Hoagland nutrient solution. When the third leaf emerged, one seedling was maintained in each pot.

The experiment included four treatments: *Fusarium graminearum* inoculation with (+K+F) or without (–K+F) potassium amendments, and two controls without *Fusarium graminearum* inoculation (+K–F, and –K–F). Plants in the latter two treatments were isolated in a separate greenhouse compartment. Potassium was added with the form of KNO_3 (5 mmol l^{-1}). In the -K treatment, NH_4NO_3 was used to balance the nitrogen concentration.

Inoculation

Roots were inoculated by irrigating the spore suspension (concentration of $5\text{-}10\times10^4$ spores ml^{-1}) into quartz sand at the sixth leaf stage (10 ml $plant^{-1}$, 35 days after germination). The infected roots were harvested at 0, 12, 24, 48 and 96 h post-inoculation and stored at -80°C for subsequent analysis.

Assays of enzymes activities in roots

Root segments (0.5 fresh weight) were extracted in 4 ml of extraction buffer containing 0.2 mol l^{-1} borate buffer (pH 8.8), 5.0 m mol l^{-1} β-mercaptoethanol and 0.1 g PVP. The extract was

centrifuged at 10,000 g for 25 min at 4 °C. PAL (EC 4.3.1.5) was assayed by measurement of the formation of trans-cinnamic acid from L-phenylalanine at 290 nm (Lisker et al. 1983). POD (EC 1.11.1.7) and PPO (EC 1.14.18.1) activity was assayed following the method of Moerschbachert et al. (1988).

Real-time quantitative reverse transcriptase PCR (Q-RT-PCR)

Real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif.). The forward and reverse primers for Q-RT-PCR were designed from the differentially expressed clones using the Primer 5 software (Table 1). A set of maize actin RNA primers was also designed for use as an endogenous control. The PCR reaction was performed using SYBR Premix Ex Taq^{TM} (Takara, Japan). Quantification of the target gene expression was carried out with comparative C_T method (Livak and Schmittgen 2001).

Table 1. Nucleotide sequences of the primers used in Q-RT-PCR.

Genes	Forward primers(5'→3')	Reverse primers(5'→3')
act	cctcaccgaccacctaat	tgaacctttctgacccaat
pod	tccaagaacctcactatcg	gtgttcgggaagaactgg
pal	aaggtgttcgtcggcatcag	gaagaaagagcaacgccaca
ppo	aagcgttgcaggtaggcc	ccgattcttgatggtggg

Evaluation of disease severity

Disease development were scored 15 d after inoculation. A unit score of 0 to 5 was assigned to each disease rate, with 0 indicating no rot, 1 = 25%, 2 = 25-50%, 3 = 50-75%, 4 = 75-100%, and 5=100% of root area rotted (Ahmad et al. 1996, Moreno-Gonzalez et al. 2004). The disease index was calculated by the following equation (Fang 1998).

$$\text{Disease index} = \frac{\sum (\text{no. of plants examined} \times \text{unit score})}{\text{total no. of plants examined} \times \text{highest score}} \times 100 \quad (5)$$

Results

Effect of K on disease incidence

The disease index was significantly lower for plants in the +K+F treatment than that in –K+F treatment, indicating that potassium application helped increase the root resistance towards the pathogen. Moreover, the shoot length and the fresh weights of both shoots and roots were higher in potassium treated plants. After inoculation, the length and fresh weight of shoots and roots were lower in the inoculated treatment compared to non-inoculated treatment, but the differences were not significant (Table 2).

Table. 2. K effect on disease index and growth of maize

Treatment	Disease index	Length (cm)		Fresh weight (g plant^{-1})	
		Shoot	Root	Shoot	Root
+K+F	11.7 b	62.8 a	11.3 a	9.2 a	3.1 a
+K–F	—	63.8 a	11.9 a	9.9 a	3.6 a
–K+F	45.8 a	42.7 b	10.8 a	2.8 b	1.2 b
–K–F	—	44.0 b	10.8 a	2.8 b	1.4 b

Means followed with the same letter with the same row are not significantly different according to a LSD test at the 5% probability level.

Potassium content of maize root and leaf

Potassium treatment led to significantly higher potassium concentrations in both roots and leaves (Fig. 1). For the leaves of inoculated plants, the potassium concentration was not different from that in non-inoculated plants. However, the potassium concentration in inoculated roots was higher than that in the non-inoculated roots in either potassium addition or omission treatments. These results indicated that maize roots tended to take up more potassium when inoculated with *F. graminearum* and this might be related to the high resistance to stalk rot.

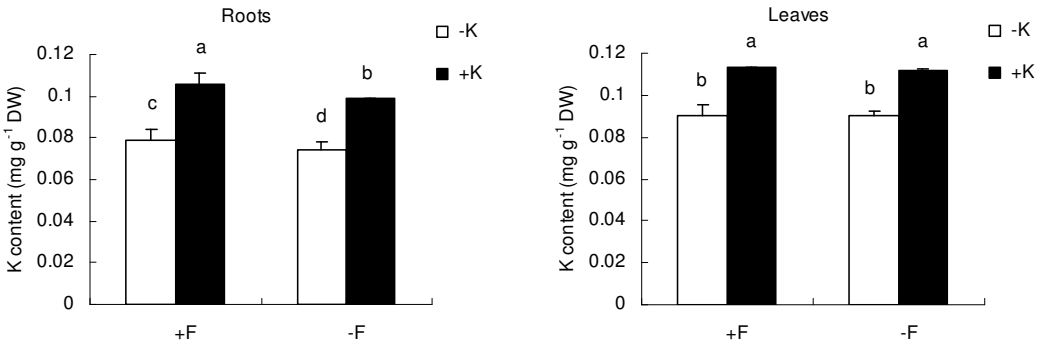

Fig. 1. Potassium content in roots and leaves of maize. Within different treatments, data following different letters were significantly different according to LSD test at 5% probability level. Error bar indicates ±SE of the mean, n=3.

PAL activity and gene expression

Treatment with potassium achieved higher PAL enzyme activities than that without potassium addition before inoculation (0 h). After inoculation, the PAL activity first decreased (24 h) and then increased. In the +K treatment, the peak of PAL activity was higher in inoculated (+F) than that in non-inoculated (–F) treatments. However, in the –K treatment, the enzyme activity showed almost no response to inoculation. This suggested that potassium addition could help increase the inherent PAL activity (Fig.2A & 2B).

No significant differences on *pal* transcripts were observed between +K+F and –K+F treatments, but in the –F treatments, *pal* expressions were lower than in the +K treatments (Fig. 2C & 2D). Pathogen induced higher *pal* expression compared with non-inoculated ones (-F), especially in the +K treatment.

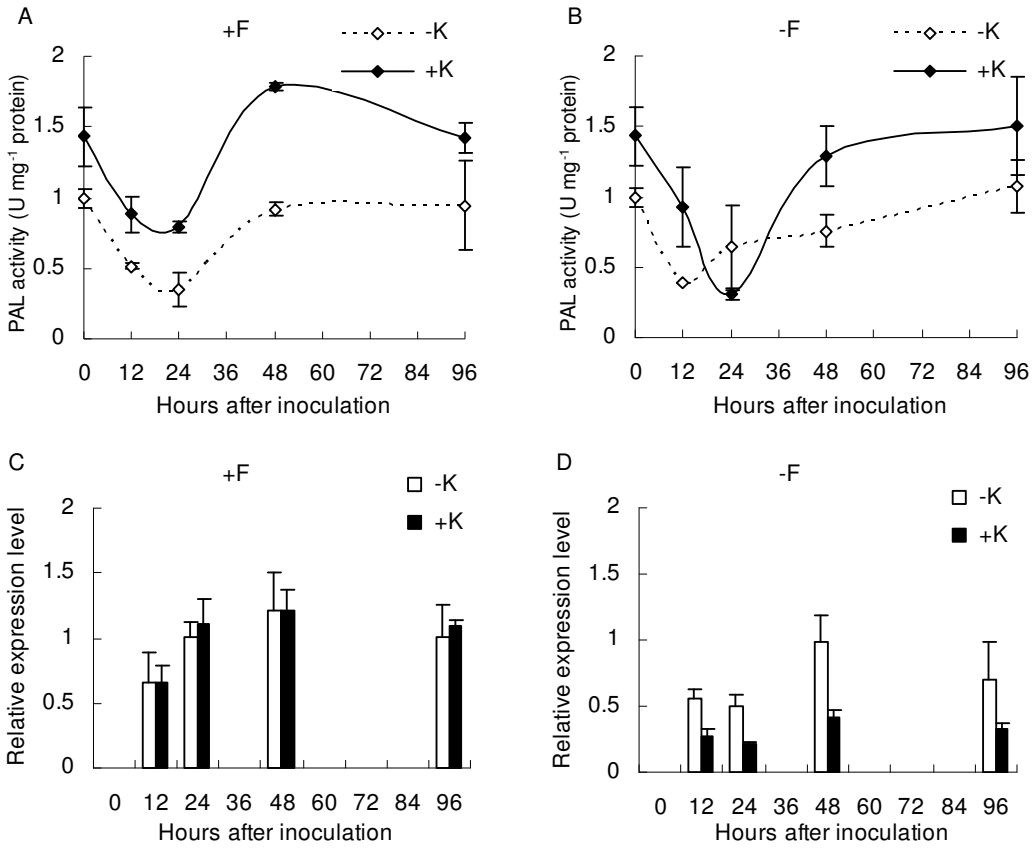

Fig. 2. PAL (phenylalanine ammonia-lyase) activity in response to potassium in maize root. (A) infection with *F. graminearum* (+F), (B) non-infection (–F) and *pal* gene expression patterns in response to potassium by using Q-RT-PCR. (C) infection with *F. graminearum* (+F), (D) non-infection (–F). Error bar indicates ±SE of the mean, n=3.

POD activity and gene expression

Changes in POD activity in response to inoculation were completely reversed in +K and –K treatments (Fig. 3A & 3B). After inoculation, POD activity in the +K treatment was enhanced significantly and reached a peak at 24 h, and then began to drop. Conversely, in the -K treatment, POD activity dropped and reached a minimum at 48 h. Before inoculation, POD activities were lower in +K treatment than in the -K treatment, which could be caused by high ROS (reactive oxygen species) production due to potassium deficiency.

In the +K treatment, *pod* expression showed an important increase during the first 12 h; the level of *pod* transcripts increased to approximately 14 times higher than that at 0 h, after which, expression slightly decreased until 96 h, when it exhibited a weak induced peak of nearly eight times higher than at 0 h. However, in –K treatment the expression peak appeared 96 h, which was about 10 fold over that at 0 h (control) (Fig. 3D).

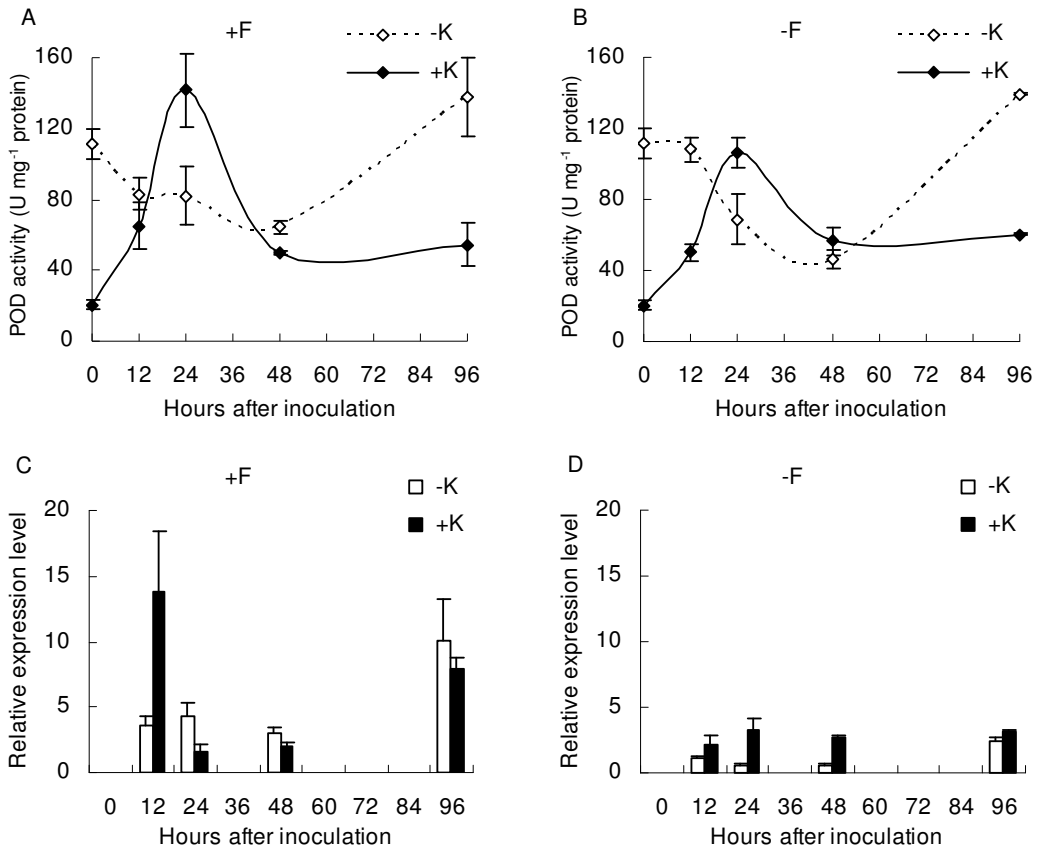

Fig. 3. POD (peroxidases) activity in response to potassium in maize root. (A) infection with *F. graminearum* (+F), (B) non-infection (–F) and *pod* gene expression patterns in response to potassium by using Q-RT-PCR. (C) infection with *F. graminearum* (+F), (D) non-infection (–F). Error bar indicates ±SE of the mean, n=3.

PPO activity and gene expression

Before inoculation, no differences existed among all the four treatments for PPO activities. However, after inoculation, the activities of PPO in the +K treatment were significantly higher than the –K treatment at 12 h, 24 h, and 48 h, and then dropped back to the original 0 h level at 96 h. In the -K treatment, PPO activity showed little change (Fig. 4A, B).

In the +K treatment, the transcripts of *ppo* peaked at 12 h (4.5-fold increase over 0 h), and then decreased (Fig. 4C). In the –K treatment, the transcript levels of *ppo* were lower after inoculation (below 1 fold) as compared to those before inoculation (1 fold) (Fig. 4C & 4D). These results demonstrated that potassium significantly increased *ppo* expression, especially when root was inoculated with the pathogen (Fig. 4C & 4D).

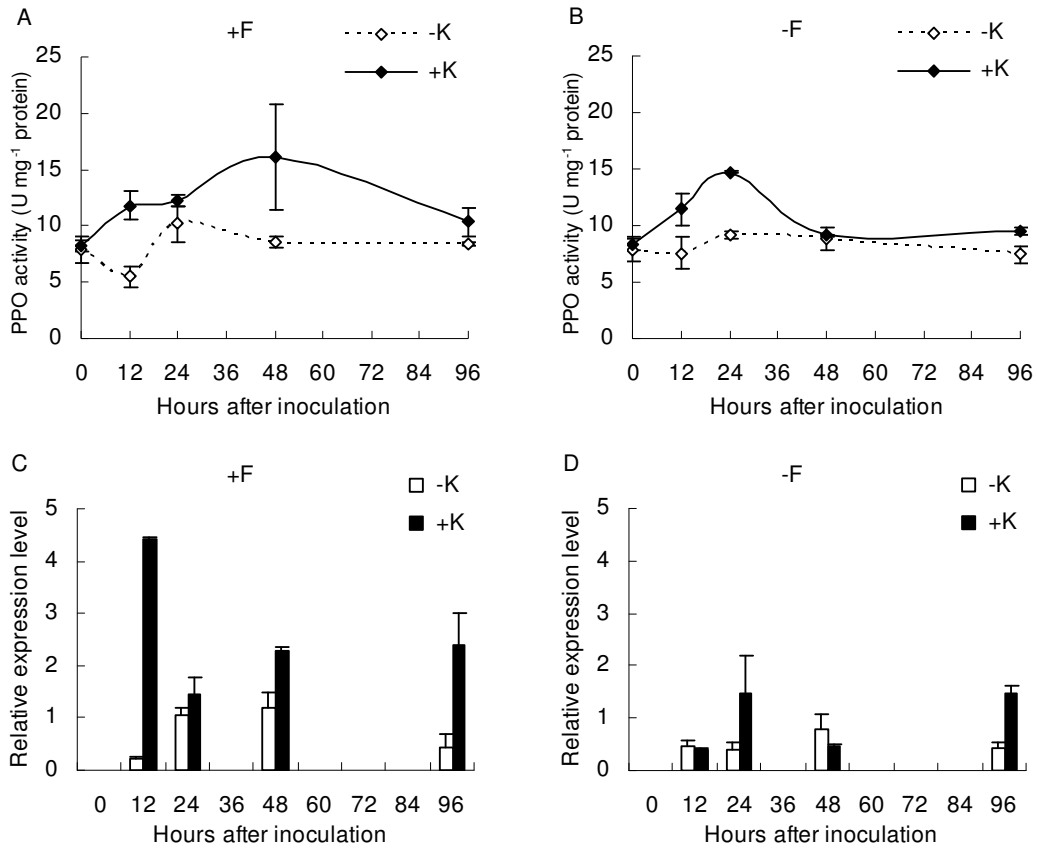

Fig. 4. PPO (polyphenol oxidases) activity in response to potassium in maize root. (A) infection with *F. graminearum* (+F), (B) non-infection (–F) and *ppo* gene expression patterns in response to potassium by using Q-RT-PCR. (C) infection with *F. graminearum* (+F), (D) non-infection (–F). Error bar indicates ±SE of the mean, n=3.

Discussion

The ability of potassium to enhance the resistance of maize against stalk or root rot has been described previously (Thayer and Williams 1960; Bullock 1990). The results obtained from the current study clearly revealed that potassium could decrease the disease incidence (Table 2). Besides, we discovered that the potassium content in infected roots was higher than in non-infected roots (Fig. 1). This provides evidence that K may participate in the response to disease resistance.

The biochemical mechanisms of plant disease resistance are complex. In several host-pathogen systems, PAL activity has been shown to increase in incompatible interactions (Ramamoorthy et al. 2002, Goâmez-vâsquez et al. 2004). In this study, we showed that higher PAL activity was achieved with potassium application than that without potassium application, which was in accordance with the previous reports.

In regard to *pal* gene expression, Sharan et al. (1998) pointed out that marked increases in PAL synthesis and corresponding mRNA levels occur in response to microbial or endogenous elicitors in many plant-pathogen systems. No significant differences on *pal* transcripts occurred whether potassium was added or not to inoculated roots, but *pal* transcripts were lower in uninoculated roots with potassium compared to those without potassium (Fig. 2). By comparison,

pal transcripts increased by pathogen was higher in +K than in –K. Thus, the current study implies that potassium could improve PAL activity in the inoculated root of maize and regulate the induced transcript of the *pal* gene.

POD and PPO have been implicated in cellular protection and disease resistance. Mohammadi et al. (2002) observed a significant increase in POD specific activity in heads of wheat cultivars following the inoculation with *F. graminearum* conidia, and this increase was demonstrated in both resistant and susceptible cultivars. Wang et al. (2006) reported that POD activity was positively correlated with plant resistance. The present study showed completely different response patterns in the +K and –K treatments. After inoculation, in the +K treatment, POD activity was enhanced very rapidly within 24 h of inoculation with F. graminearum, while in the –K treatment, its activity decreased first and then increased slowly at 48 h. In addition, the induced POD activity was also higher with potassium addition as well.

Moreover, potassium increased the expression and activity of POD. Although the status of POD activity was also enhanced in potassium deficient root, the enhancement occurred too late to prevent disease development. Following elicitation, pod expression was triggered earlier and lasted for longer than that of pal in +K treatment, which was different with Goâmez-vâsquez et al. (2004) research. We deduced that the K effect on the phenol metabolism which POD participated was to promote the development of thicker outer walls in host cells, thus preventing disease attack (Dordas 2008), and that these affects were stronger than those on PAL.

Interestingly, the activities of POD were naturally present in maize and seemed to be constitutive in nature. The rapid induction of *pod* was not specifically detected in the resistant cultivar, but was also shown in the susceptible plant (Kuroda et al. 2006). The *pod* gene in young roots might contribute to the basal resistance. The increase in POD activity was important in the defense mechanism but it was not a determinant of the defense mechanism (de Armas et al. 2007). Rather, the mechanism, time and trend of its maximum induction suggested a significant role in governing plant resistance to disease (Gogoi et al. 2001). In our study, the background level of POD activity was higher in the –K than in the +K treatment, but the potassium abundant in the root had a higher and earlier induction.

Due to its conspicuous reaction products and its wound and pathogen inducibilities, PPO has frequently been suggested to participate in plant defense against pests and pathogens (Thipyapong et al. 1995, Thipyapong and Steffens 1997). In this study, we observed a significant increase in PPO activity in +K+F roots of maize, which peaked at 48 h, and then decreased. In –K treatment, PPO activity was little changed (Fig. 4). Moreover, potassium enhanced the induced PPO activity whether maize was injured by the pathogen or not. Li and Steffens (2002) indicated that increasing constitutive levels of PPO in tomato leaves conferred the ability to oxidize the pool of pre-formed phenolics faster in response to the bacterial pathogen in the infection process. Our results showed that the transcript accumulation increased after pathogen inoculation in roots with added potassium, but not in roots with no added potassium. The accumulation of *ppo* mRNA quickly increased to more than 4-fold only at 12 h in +K+F treatment. Based on the current study, we conclude that potassium might play a role in regulating the expression of *ppo* or ameliorating the activity of PPO.

In this paper, physiological and molecular parameters have given preliminary information about the resistance mechanism of maize to stalk rot as related to phenol metabolism. The changes in PAL, POD and PPO enzyme levels/activities and the accumulation patterns of the *pal, pod* and *ppo* transcripts in maize roots, as related to the resistance mechanism of stalk rot induced by potassium deficiency, had not been described previously. The results presented in

this study extend the current knowledge of such cross talk between plant nutrition and plant pathology. The research also opened entirely new avenues for the fine-tuning of potassium fertilizer application and disease control to improve crop health and quality, by reducing the input of chemicals into agriculture, thereby supporting efforts to achieve an economically and environmentally sustainable increase in food production.

Acknowledgements

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