Full Length Research Paper

Effect of nitrogen supply on rhizobacterial propagation and soil enzyme activity in pot soil cultivated with *Phytophthora infestans*-infected potato plants

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We previously demonstrated that providing potato plants with an optimum nitrogen supply (in the form of urea; 135 kg hm^{-2}) produces high crop yields and increased resistance to late blight. However, the mechanisms underlying these responses have not been well characterized. In this study, we examined the effects of various levels of nitrogen fertilization (four levels: N1, 45 kg hm^{-2}, N2: 90 kg hm^{-2}, N3: 135 kg hm^{-2} and N4: 180 kg hm^{-2}) on soil bacterial growth, community diversity and soil enzyme activity. We found that application of the optimum amount of nitrogen promoted maximum levels of bacterial growth and community diversity development compared with the other treatments. In addition, the highest activities were detected for soil enzymes such as urease, invertase and acid phosphatase (but not catalase) under N3 conditions but not under N1, N2 or N4 conditions. These results suggest that proper nitrogen application provides soil microbes with optimum conditions for development. Thus, the optimum growth of rhizobacteria conferred by N3 treatment appears to be responsible for achieving the highest yields and strongest pathogen resistance in potato plants exposed to *Phytophthora infestans*.

Key words: Late blight, potato plant, rhizobacteria, soil enzyme, urea fertilizer.

INTRODUCTION

Nitrogen fertilizer is added to soils to stimulate metabolism in plant chloroplasts, which are responsible for the process of photosynthesis (Radin, 1983). Two forms of nitrogen are commonly used for plant growth (Ingestad, 1977). The first

form is natural nitrogen, which is found in animal matter and decaying plants. The second form is commercially synthesized nitrogen fertilizer in the form of ammonium or nitrate. Ammonium-based fertilizers bond securely with the soil and the nitrogen in these fertilizers is slowly released to the plant. The nitrogen in nitrate-based fertilizers is quickly absorbed by the plant, but it is easily washed away by water via a process known as leaching (Kraiser et al., 2011).

Microorganisms in soils are important because they affect the structure and fertility of various soils. Bacteria, the most abundant microbe in the soil, play many important roles involving numerous biochemical processes. One important trait of bacteria is their role in the process of nitrogen fixation, the conversion of atmospheric nitrogen into nitrogen-containing compounds that are utilized by plants (Burns and Hardy, 1975).

Microbial diversity in soils is affected by various factors including anthropogenic activities. The activity and growth of microbial communities increase in response to organic matter amendment. For example, application of chemical fertilizer or manure increases the number of total and spore-forming bacteria, actinomycetes and fungi in the soil (Kanazawa et al., 1988). Belay et al. (2002) also reported that nitrogen/phosphorus/potassium have a direct effect on the number of bacteria, actinomycetes and fungi in soil used for maize cultivation. In addition, variations in microbial populations in sorghum soils might be attributed to the effect of chemical fertilization (Gryndler et al., 2003).

Successful infection of plants by pathogens requires the efficient use of nutrient resources available in host tissues (Dordas, 2008). Over- or under-application of nitrogenous fertilizer can result in a higher susceptibility of plants to fungal pathogens (Snoeijers et al., 2000; Hoffland et al., 2000). *Phytophthora infestans* is an important oomycete pathogen of potato plants that causes late blight disease, which can lead to a significant reduction in tuber yields (Moller et al., 2006).

Interestingly, numerous studies have shown that insect resistance and abiotic stress tolerance are often not compatible with rapid growth. Plants selected for high biomass accumulation may also have low natural resistance to environmental stress and vice versa (Coley et al., 1985; Hamilton et al., 2001; Zhu, 2001; Züst et al., 2011). However, rapid growth and increased abiotic stress tolerance have been observed simultaneously in transgenic *Arabidopsis thaliana* (Deng et al., 2013).

We previously demonstrated that providing potato plants with an optimum nitrogen supply (135 kg hm^{-2}) can increase plant yields and pathogen resistance under *P. infestans* exposure (Jin et al., 2014). However, the mechanism underlying this process has not been well characterized. In the current study, we addressed two related questions. First, how does nitrogen supply affect soil bacterial diversity and density? Second, does nitrogen-affected soil enzyme activity also contribute to increased yield and pathogen resistance in potato? The results of this study provide insights into the contribution of soil bacterial propagation to plant pathogen resistance and yield under various nitrogen supply conditions.

MATERIALS AND METHODS

Plant growth and nitrogen treatment

This study stems from previous research described in the African Journal of Agricultural Research (Jin et al., 2014). The experiment was performed under greenhouse conditions in Daqing City, Heilongjiang Province, China, using 5 L pots with an effective depth of 40 cm and a hole at the bottom to drain excess water. The pots contained 4 L of nutrient soil (Table 1).

The experiment was arranged in a completely randomized design with five replications. Treatments comprised four levels of N supply (designated N1, N2, N3 and N4). Nitrogen (in the form of urea) was added at 20 days after emergence, and an amount equivalent to 45 (N1), 90 [(N2), the mean level of N supply in Northeast China for potato cultivation)], 135 (N3) and 180 kg-hm^{-2} (N4) was applied, respectively. Each pot was considered to represent an experimental unit. The potato cultivar Kexin 1 from Keshan Farm, Potato Research Institute, Heilongjiang Province, China was used in this experiment. Kexin 1 (registration number: GS05009-1984) is a hybrid potato cultivar that was bred and selected in China in 1958 from a cross between 374-128 (maternal line) and *Epoka* (paternal line). In this study, the potato cultivar was planted on 15 June, 2011 at a depth of 15 cm using one seed tuber per pot with a diameter of 45-50 mm, containing vigorous shoots. All experiments were conducted in a greenhouse under a 16:8 h light/dark cycle (200 µmol photon m^{-2}s^{-1}) at a temperature of 22–25°C and 50–70% relative humidity. The potato seedlings were inoculated with the pathogen *P. infestans* prior to soil sample collection (Jin et al., 2014).

Table 1. Chemical properties of the soils used in this study.

Parameter	Sampled soil (0-10cm) in this experiment
pH value	8.28
Total salt content (g kg^{-1})	3.09
Organic matter (g kg^{-1})	12.80
Alkaline solved N (mg kg^{-1})	60.80
Effective P (mg kg^{-1})	40.30
Effective K (mg kg^{-1})	121.60

Soil sample collection and treatment

Top soil samples (0–10 cm) were collected from pots containing cultivated, inoculated potatoes. The soils were air dried and sieved (2 mm), and 1.5 kg of each soil sample (dry weight equivalent) was placed into a 5 L plastic container. The water content was adjusted to 70% water holding capacity by the addition of deionized water. Aeration was provided through two 5 mm diameter holes in the lid of the container. Soil moisture was adjusted every 4 days based on weight loss. Soils were sampled after potato cultivation for 40 (seedling stage: designated S), 60 (tuber formation stage: designated T1), 80 (tuber expansion stage: designated T2) and 120 (starch accumulation stage: designated T3) days by removing

approximately 200 g of soil from the entire depth of each container. After each sampling, the soils in the containers were compacted to an approximate bulk density of 1 g cm^{-3}.

Microbial counts

Ten grams of each soil sample was added to 90 mL of 0.1% (w/v) of sodium pyrophosphate solution. After homogenization for 30 min, the solution was decimally diluted (10^{-1} to 10^{-7}), and aliquots of the resulting solutions were plated onto appropriate culture media. After incubation at 30°C for up to 5 days, the colony forming units (CFU) were counted (Vieira and Nahas, 2005).

Soil enzyme extraction and measurement

Soil enzyme activities were assayed in triplicate air-dried samples as described by Shi et al. (2008). Briefly, urease activity was assayed using urea as a substrate. After the soil mixture was incubated at 37°C for 24 h, the produced NH$_3$-N was measured using a colorimetric method; urease activity is expressed as mg NH$_3$-N g^{-1}day^{-1}. Invertase activity was assayed using sucrose as a substrate following incubation at 37°C for 24 h. The produced glucose was measured using a colorimetric method; invertase activity is expressed as mg glucose g^{-1}day^{-1}. Acid phosphatase activity was assayed using sodium phenolphthalein phosphate as a substrate. Following incubation at 37°C for 24 h, the liberated phenol was determined colorimetrically; acid phosphatase activity is expressed as mg phenol g^{-1}day^{-1}. Catalase activity was assayed using H$_2$O$_2$ as a substrate. The mixture was shaken for 20 min and filtered. The filtrate was titrated with 0.1 M KMnO$_4$; catalase activity is expressed as mg KMnO$_4$ g^{-1}min^{-1}.

Rhizobacterial diversity assay

At four sampling dates (days 40, 60, 80 and 120), the structure of the soil microbial community was assessed via extraction of community DNA, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) separation. Community DNA was extracted from the soil samples using an UltraClean^{TM} Soil DNA kit (MoBio Laboratories, Inc., USA) according to manufacturer's instructions, followed by PCR amplification targeting 16S rDNA from eubacteria (forward primer F338GC [5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3'] and reverse primer R518 [5'-ATT ACC GCG GCT GCT GG-3'] (Li et al., 2008). For all amplifications, 25 μL reaction mixtures were used, containing 2.5 μL Hot Master Taq buffer (10×; Eppendorf, Germany), 2 μL dNTPs (2.5 mM), 1 μL bovine serum albumin (0.01 g mL^{-1}; first round only for actinomycetes), 1 μL each of forward and reverse primers (10 μM), 0.25 μL Hot Master Taq DNA polymerase (Eppendorf, Germany; 1.25 units) and 1 μL of template DNA (first round) or diluted PCR product from the first round (1:200; second round). The thermal cycling conditions were as follows: F338GC–R518: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, with a final extension step of 7 min at 72°C. Amplified DNA was verified by electrophoresis of aliquots of PCR mixture (5 μL) in a 1% agarose gel in 1× TAE buffer. The DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) was used for DGGE. Six microliters of PCR product plus 4 μL of water were loaded onto an 8% (w/v) acrylamide gel (acrylamide/bis solution, 37.5:1) with a linear chemical gradient of 40–70% (100% = 7 M urea and 40% [v/v] formamide). The gels were run in 1×TAE buffer (preheated to 60°C) for 10 min at 200 V followed by 16 h at 80 V. The gels were silver stained to detect DNA using a standard protocol (Sanguinetty et al., 1994). The gels

were dried overnight at 60°C before being scanned using a GS-700 Imaging Densitometer (Bio-Rad, USA).

Data analysis

All data were analyzed using SPSS 13.0 software. Means followed by the same letter were not significantly different (Duncan's multiple range test $P < 0.05$). For each treatment, five replicates were analyzed. DGGE patterns were analyzed by cluster analysis according to Ward (1963) using Quantity One 1-D Analysis Software (Version 4.5.2; Bio-Rad, USA).

RESULTS

The effect of nitrogen on rhizibacterial growth

In this experiment, rhizobacterial growth in topsoil (1–10 cm) was measured during the four growth stages (S, T1, T2 and T3) of potato plants (Figure 1). N3 treatment had the greatest promotive effect on rhizobacterial propagation compared with the other nitrogen levels. As the nitrogen supply increased, the number of rhizobacteria increased, followed by a decrease. For example, an approximately 2.5-fold increase in growth was observed during the T1 growth stage following N3 treatment compared with N1 treatment. In addition, a similar pattern was observed as plant growth progressed: the population increased, followed by a decrease (Figure 1). For example, the N2, N3 and N4 treatment groups displayed higher levels of rhizobacteria at T1 than at the other growth stages (Figure 1).

The effect of nitrogen on rhizobacterial diversity

We investigated the diversity of soil bacteria using DGGE (Table 2 and Figure 2). As shown in Table 2 and Figure 2, the soil exhibited the most abundant bacterial diversity in the N3 treatment group compared with the N1, N2 and N4 groups irrespective of growth stage. For example, at the T1 growth stage, an approximately 30, 9 and 11% increase in the number of bands in the DGGE gel was detected in the N3 treatment group compared with the N1, N2 and N4 treatment groups, respectively (Table 2). In addition, the richness index and diversity index (*Dsh*) were significantly higher in the N3 treatment group than in the three other treatment groups (Table 2). However, there was no significant difference in the homogenous degree index (*Jsh*) between the N3 group and N2 or N4 during the first three growth stages (Table 2). Similar patterns were also observed during the other growth stages, that is, S, T2 and T3 (Table 2 and Figure 2).

The effect of nitrogen on soil enzyme activity

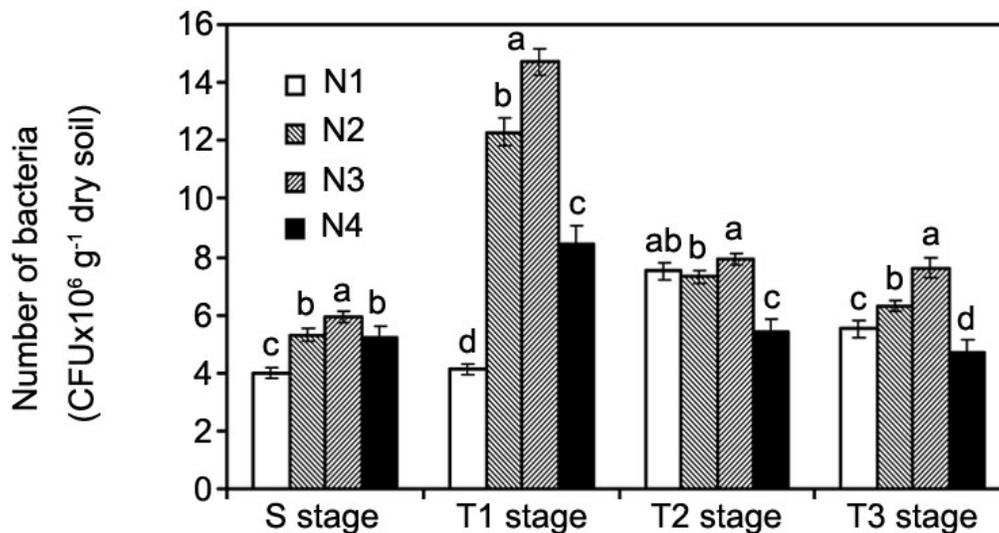As shown in Figure 3A, there were significant differences in urease activity between the groups during the S stage

Figure 1. The effects of nitrogen on bacterial growth. Effects of nitrogen supply on soil bacterial growth in the rhizosphere of potato plants. Data are presented as the means of five independent experiments. Means followed by the same letter were not significantly different between the four nitrogen supply levels (Duncan's multiple range; test P < 0.05). Bars show mean ± SE (n = 5).

Table 2. The richness index, diversity index (*Dsh*) and homogeneous degree index (*Jsh*) in the rhizosphere soils of potato at four growth stages under different nitrogen supply conditions.

Treatment		*Band number*	*Richness index*	*Dsh*	*Jsh*
	N1	36	10a	1.09 ± 0.01b	0.59 ± 0.013b
S	N2	39	10a	1.25 ± 0.03a	0.64 ± 0.016a
	N3	42	11a	1.31 ± 0.02a	0.70 ± 0.009a
	N4	35	10a	1.16 ± 0.01b	0.67 ± 0.010a
	N1	37	13b	1.55 ± 0.01c	0.94 ± 0.013b
	N2	44	12b	2.15 ± 0.03b	0.96 ± 0.016a
T1	N3	48	15a	2.54 ± 0.02a	0.98 ± 0.009a
	N4	43	12b	2.21 ± 0.01b	0.97 ± 0.010a
	N1	36	12b	1.49 ± 0.01b	0.76 ± 0.013b
	N2	38	12b	1.55 ± 0.03b	0.80 ± 0.016b
T2	N3	41	14a	2.28 ± 0.02a	0.92 ± 0.009a
	N4	36	13a	2.06 ± 0.01a	0.89 ± 0.010a
	N1	32	11a	1.19 ± 0.01b	0.62 ± 0.013b
	N2	36	12a	1.25 ± 0.03a	0.63 ± 0.016b
T3	N3	42	13a	1.28 ± 0.02a	0.68 ± 0.009a
	N4	39	12a	1.06 ± 0.01b	0.59 ± 0.010b

(N1 < N2 < N3), but this difference decreased during the three remaining growth stages. For example, an approximately 50, 60 and 10% increase in urease activity was detected in N3 when compared with N1, N2 and N4, respectively, at T2. In addition, N3 treatment also significantly increased urease activity compared with N1, N3 and N4 treatment during the S, T1 and T2 growth stages

(Figure 3A).

Similar patterns were observed for invertase activity in response to nitrogen treatment (Figure 3B). The N3 treatment group has the highest invertase activity compared with N1, N2 and N4 (Figure 3B). For example, an approximately 90, 10 and 20% increase in enzyme activity was detected in N3 when compared with N1, N2
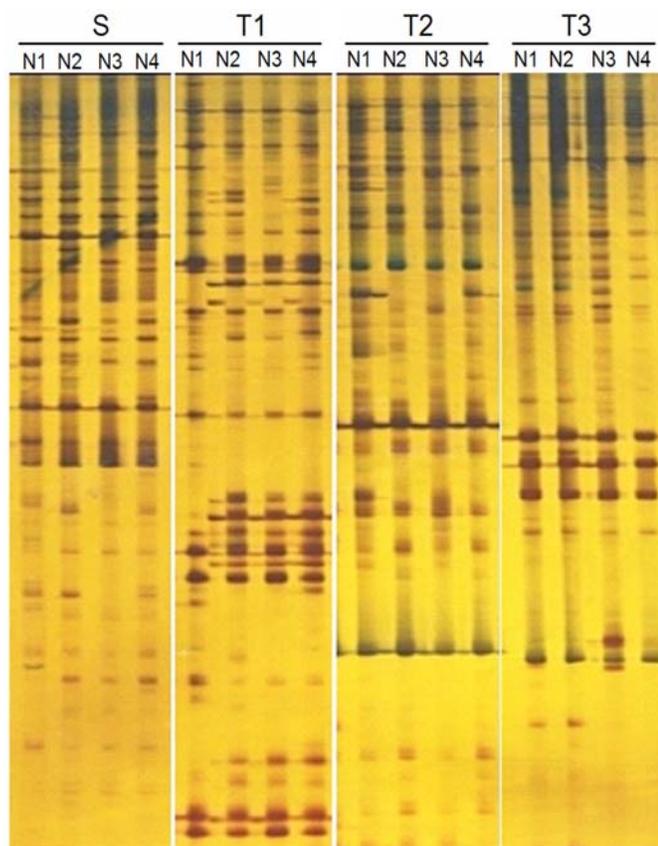
Figure 2. PCR-DGGE fingerprints of bacteria in the rhizosphere soil of potato plants. Lanes show PCR-DGGE fingerprints of bacteria in rhizosphere soil of N1-, N2-, N3- and N4-treated potato plants during growth stages S, T1, T2 and T3, respectively.

and N4, respectively, at the T1 growth stage (Figure 3B).

The highest acid phosphatase activity in the topsoil was also detected for the N3 treatment group when compared with the three other treatment groups (Figure 3C). However, the lowest enzyme activity was observed for all treatment groups at T2 when compared with S, T1 and T3 (Figure 3C). For example, the acid phosphatase activity was approximately 50, 35, 20 and 15% lower in N1, N2, N3 and N4 during T2 when compared with the S growth stage, respectively (Figure 3C).

By contrast, a different pattern was detected for catalase activity compared with urease, invertase and acid phosphatase activity. For example, at T1, CAT activity increased approximately 10, 60 and 165% in the N2, N3 and N4 treatment groups, respectively, over N1 control levels (Figure 3D). In addition, CAT activity tended to increase during the course of plant growth (Figure 3D).

DISCUSSION

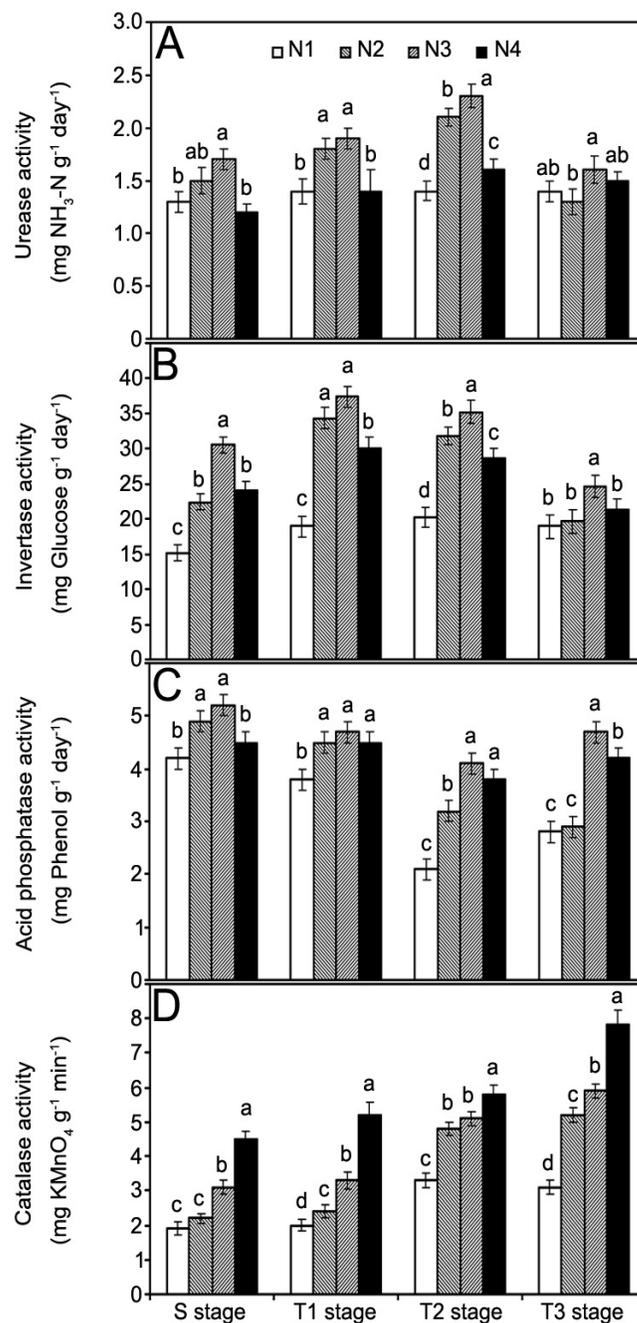We previously demonstrated that potato plants supplied

Figure 3. The effects of nitrogen on soil enzyme activity. Effects of nitrogen supply on the activities of soil enzymes urease (A), invertase (B), acid phosphatase (C) and catalase (D) in the rhizosphere of potato plants. Data are presented as the means of five independent experiments. Means followed by the same letter were not significantly different between the four nitrogen supply levels (Duncan's multiple range; test P < 0.05). Bars show mean ± SE (n = 5).

with N3 (135 kg hm^{-2}) levels of nitrogen have the highest yields and strongest pathogen resistance under late blight exposure (Jin et al., 2014). In the current study, we investigated whether this interesting phenomenon can

partially be attributed to the effect of nitrogen on nitrogen-affected soil microorganisms, especially rhizobacteria.

Our data show that N3 treatment of soil under potato cultivation promoted the highest level of rhizobacterial growth (Figure 1). To study the bacterial diversity in the soil, we utilized a reliable method: the DGGE finger-printing technique. Analysis of the fingerprint banding pattern supports the notion that N3 treatment supplies the soil sphere with the optimum nutrient availability conditions for rhizobacterial community development and diversity formation (Figure 2 and Table 2). For example, the highest number of bands (42, 48, 41 and 42 bands during the S, T1, T2 and T3 growth stages, respectively) was detected under N3 conditions but not under other nitrogen supply conditions (Table 2). In addition, the highest activities of soil enzymes (urease, invertase and phosphatase) were also observed in the N3 group compared to the other treatment groups (Figure 3). These data indicate that rhizobacteria exhibit maximum activity in the rhizosphere of potato plants under optimum nitrogen application conditions.

Soil microbes adapt to changes in environmental conditions through changes to their proliferation and metabolism (Marshall et al., 2000). Soil microbial activity is regarded as one of the most important parameters of soil microorganisms and has been suggested to serve as an indicator of soil health (Brussaard et al., 2007). In the present study, the number of bacteria increased signifi-cantly in soil in response to the addition of N3 (but not N1, N2 or N4) levels of urea. The possible reasons for this response are presented below.

The microbial community is involved in numerous ecosystem functions in soil, such as nutrient cycling and organic matter decomposition (Hueso et al., 2011), and it is a more external stress-responsive component of the terrestrial ecosystem than plants or animals (Panikov, 1999). Soil microorganisms synthesize and secrete extra-cellular enzymes, which constitute an important part of the soil matrix. Enzymes play an important role in soil nutrient cycles. Consequently, factors influencing soil microbial activity also affect the production of enzymes that control nutrient availability and soil fertility (Hueso et al., 2011). Urease is one of the most commonly assayed soil enzymes because it greatly influences the trans-formation and fate of urea, an important fertilizer. Con-sequently, soil urease activity has attracted considerable attention due to the increased use of urea as a fertilizer to increase soil productivity. In this study, the highest urease activity was detected in soil under N3 level fertilization, indicating that an optimum urea supply can be useful for enhancing the nitrogen cycle in soil (Figure 3A). Invertase is an extracellular enzyme that catalyzes the hydrolysis of sucrose into glucose and fructose. The high levels of invertase activity observed in the present study may be ascribed to high total organic carbon levels, and they also suggest that soil fertility increases in soil under N3 fertilization (Figure 3B). Phosphatase enzymes

are used by soil microbes to access organically bound phosphate nutrients. In this study, the highest acid phosphatase enzyme activity was observed in the N3 treatment group, showing that a proper supply of nitrogen fertilizer can satisfy the biological demands of soil microbes by enabling them to access the optimum levels of phosphates in the soil (Figure 3C). In addition, catalase uses a two-electron transfer mechanism to scavenge H_2O_2 by splitting this molecule into molecular oxygen and water, thereby protecting cells from damage caused by reactive oxygen species (Guwy et al., 1999). Increased catalase activity was detected in soils containing urea, suggesting that increased H_2O_2 levels may be induced by the application of high levels of urea (Figure 3D).

Rhizobacteria, especially plant growth-promoting rhizobacteria (PGPR), are root-colonizing bacteria that form symbiotic relationships with many plant species. PGPR can increase the availability of nutrients through the solubilization of unavailable forms of nutrients and via the production of siderophores, which facilitate nutrient transport (Mantelin and Touraine, 2003; Glick et al., 2007). Phosphorus, a limiting nutrient for plant growth, can be plentiful in soil but is most commonly found in insoluble forms. Organic acids and phosphatases released by rhizobacteria in the plant rhizosphere facilitate the conversion of insoluble forms of phosphorus to plant-available forms, such as $H_2PO_4^-$. In addition, PGPR can enhance plant growth by reducing the harmful effects of plant pathogens on crop yield.

Rhizobacteria can also help control plant diseases caused by other bacteria and fungi. Disease is suppressed through induced systematic resistance and through the production of anti-fungal metabolites (Barriuso et al., 2008). For example, Barriuso et al. (2008) found that PGPR protect *A. thaliana* against various types of pathogens by simultaneously activating the salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) signaling pathways. SA plays an important role in basal defense of *Solanum tuberosum* against *P. infestans* which causes late blight (Halim et al., 2007). The current data suggest that N3-stimulated PGPR growth may contribute to the strong biotic resistance of potato during the plant–pathogen interaction (Jin et al., 2014). A hypo-thetical model was proposed to illustrate the mechanism underlying the nitrogen supply-mediated promotion of plant growth and pathogen resistance (Figure 4).

Some interesting conclusions can be drawn from the current study. First, the N3 level of urea (135 kg hm^{-2}) application is the optimum amount for soil microorganism growth and healthy community formation. Second, the activities of soil enzymes such as urease, invertase and acid phosphatase are highest under N3 fertilization. The results of this study suggest that providing an optimum nitrogen supply (urea in this study) can optimize soil sphere conditions for rhizobacterial growth, which contributes to the production of potato plants with the highest yields and strongest pathogen resistance.
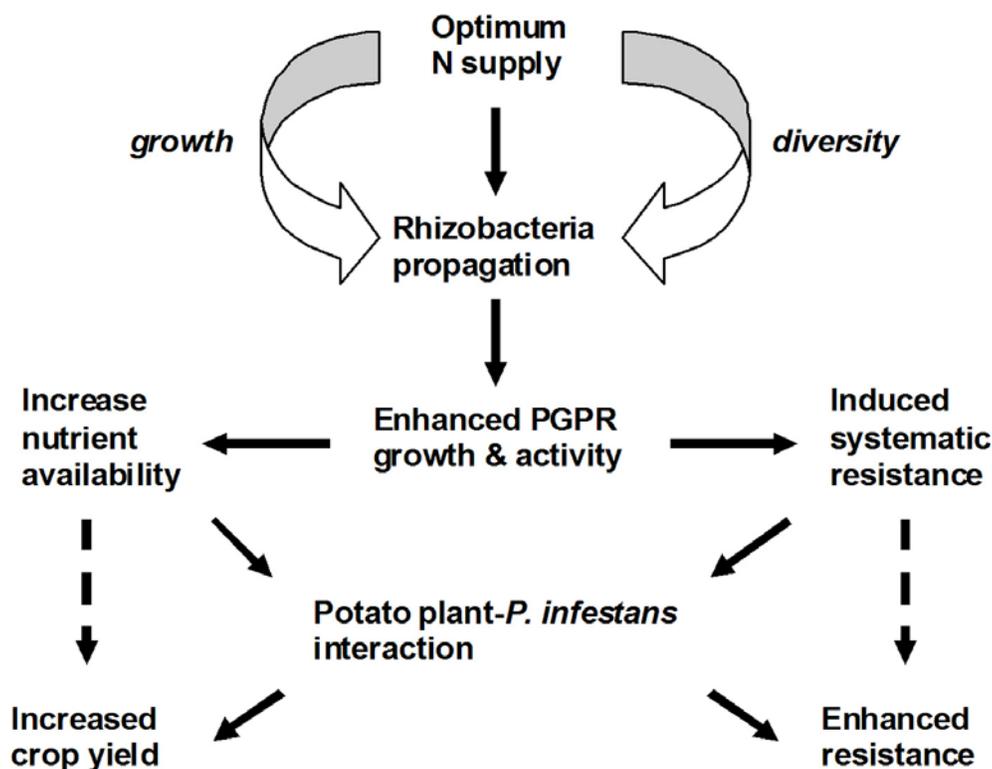
Figure 4. A hypothetical model based on plant growth and defense theory illustrating the mechanism underlying the simultaneous increased crop yields and pathogen resistance during the plant–pathogen interaction under optimum nitrogen supply conditions.

Conflict of interests

There is no conflict of interest among the authors.

ACKNOWLEDGEMENTS

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