Soil Science and Plant Nutrition (2008) **54**, 560–565 doi: 10.1111/j.1747-0765.2008.00276.x

ORIGINAL ARTICLE/SHORT PAPER

Effect of supplied phytosiderophore on ^{59}Fe absorption and translocation in Fe-deficient barley grown hydroponically in low phosphorus media

Antoine LADOUCEUR[1], Fumihito AKIHA[2] and Shigenao KAWAI[3]

[1]*The United Graduate School of Agricultural Sciences*, [2]*Laboratory of Radio Isotope and* [3]*Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan*

Abstract

Hydroponically grown barley plants (*Hordeum vulgare* L. cv. Minorimugi) under iron-deficient (–Fe) and high phosphorus (P) conditions (500 μmol L^{-1}) showed Fe chlorosis and lower growth compared with plants grown in –Fe and low P conditions (50, 5 and 0.5 μmol L^{-1}). To understand the physiological role of P in regulating the growth of plants in –Fe medium, we carried out an Fe feeding experiment using four P levels (500, 50, 5 and 0.5 μmol L^{-1}) and phytosiderophores (PS), mugineic acid. Our results suggest that plants grown in a high P medium had higher absorption activity of ^{59}Fe compared with plants grown in low P media, irrespective of the presence or absence of added PS. Translocation of ^{59}Fe from roots to shoots was not affected by the P level. The relative translocation rate of ^{59}Fe increased with decreasing levels of P in the medium. In general, the addition of PS enhanced the absorption of ^{59}Fe and its translocation. Taken together these results suggest that the lower relative translocation rate of Fe in high P plants may be induced by the physiological inactivation of Fe in the roots, and the higher absorption activity of Fe in high P conditions possibly results from the response of barley plants to Fe deficiency.

Key words: apoplastic iron, ^{59}Fe, iron deficiency, low phosphorus, phytosiderophores.

INTRODUCTION

Iron (Fe) and phosphorus (P) are two essential mineral elements for plant growth and development and deficiencies in these two elements play a major role in limiting crop yield worldwide. A deficiency in these elements in the soil may result from various reasons. For example, the chemical forms of Fe found in most soils are sparingly soluble in soil solution at biological pH, and in calcareous soils Fe concentrations in soil solution usually range from 0.1 to 10% of the total needed by the plants (Lindsay 1984). The formation of water-insoluble Fe oxides and oxyhydroxides in calcareous soils further affects the availability of Fe to plants, even when the total Fe concentration is relatively high (Hartwig and Loeppert 1993; Lindsay and Schwab 1982) in such soils. A deficiency of Fe in soil ultimately results in Fe chlorosis induced by high pH or carbonate (Römheld and Marschner 1991). Similarly, a deficiency in P, which is the second most limited nutrient for plant growth after nitrogen (Vance *et al*. 2003), results from fixation of P by Fe and Al in acidic conditions and formation of insoluble complexes by calcium in alkaline or calcareous conditions (Mengel and Kirkby 2001).

Combined deficiencies of P and other micronutrients may potentially occur in calcareous and alkaline soils. For example, most of the chickpea-growing regions of India are deficient in P as well as other micronutrients (Srinivasarao *et al*. 2006).

The ratio of Fe to P in plant tissues has been reported as one of the regulators of Fe chlorosis in some plants. Several reports have shown that a high Fe/P ratio reduces the expression of Fe chlorosis (DeKock and Alexander 1955; Ladouceur *et al*. 2006; Pushnik *et al*. 1984). Ladouceur *et al*. (2006) found that the Fe concentration in shoots of plants grown under –Fe and high or low P conditions varied within the range of the critical deficiency level of 30–50 μg g^{-1} (Römheld and Marschner 1991) or was lower than 65 μg g^{-1} dry matter

Correspondence: S. KAWAI, Faculty of Agriculture, Iwate University, Ueda 3-18-8, Morioka 020-8550, Japan. Email: skawai@iwate-u.ac.jp

Received 24 September 2007.
Accepted for publication 24 February 2008.

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(Tang *et al*. 1990). However, plants grown in low P conditions did not show Fe chlorosis (Ladouceur *et al*. 2006). It is known that –Fe symptoms in plants occur because of factors inhibiting Fe absorption and translocation or impairing its utilization in metabolic processes rather than as a result of scarcity in the environment (Brown 1961; Welch *et al*. 1991). Low P appears to activate Fe for chlorophyll synthesis in plant tissues under Fe-stressed conditions. The physiological mechanism of absence of chlorosis in low P conditions has not been well clarified.

The phytosiderophores (PS) released by graminaceous plants (Strategy II plants) are well known for their Fe acquisition capability in Fe-stressed conditions (Römheld and Marschner 1986; Takagi *et al*. 1984). Takagi *et al*. (1984) reported that the addition of PS to the medium increased Fe uptake by rice seedlings, resulting in greening of the chlorotic leaves. High P concentration in –Fe medium enhanced the release and accumulation of PS in roots (Ladouceur *et al*. 2006). Furthermore, Alam *et al*. (2005) showed that the addition of PS to the media resulted in an increase in ^{59}Fe absorption and translocation from roots to shoots in barley. Taken together these results suggest that PS is an important regulator for Fe absorption and translocation in plants. However, the roles of PS and/or P on Fe absorption and translocation in Strategy II plants under Fe stress remain elusive. To clarify the role of PS in the absorption and translocation of Fe in Fe-stressed and low P conditions, a feeding experiment was conducted using radioactive ^{59}Fe in barley plants in the presence or absence of PS in the growth medium.

MATERIALS AND METHODS

Plant culture and growth

Seedlings of barley plants (*Hordeum vulgare* L. cv. Minorimugi) were cultivated using a previously described method (Ladouceur *et al*. 2006). Plants were transplanted in bunches of three plants wrapped with sponge rubber and transferred to 10-L plastic buckets (16 bunches per bucket) filled with half-strength Hoagland–Arnon solution. After 2 days of growth in the above nutrient solution, the plants were transferred to –Fe half-strength modified Hoagland–Arnon solution (Takagi 1993) with four P levels, 500 (original concentration in the Hoagland–Arnon solution), 50, 5 and 0.5 μmol L^{-1} P. Phosphorus was supplied as NaH_2PO_4. The plants were grown in a phytotron (day/night 14/10 h; temperature 17/10°C; light intensity 280 μmol m^{-2} s^{-1}). The pH of the nutrient solutions was monitored daily and adjusted to 6.5. The nutrient solutions were renewed weekly. The plants were allowed to grow for 12 days after treatment (DAT) prior to the ^{59}Fe feeding experiment.

Chlorophyll index of the leaves

Chlorophyll content in the fourth leaves was measured in three bunches of plants at harvest using a SPAD-502 chlorophyll meter (Minolta Camera Company, Tokyo, Japan).

Source of used phytosiderophores and ^{59}Fe

Mugineic acid (one of the major PS released by the barley cultivar) was collected from the root washings of –Fe barley using a previously described method (Takagi 1993). The ^{59}Fe-radionuclide was purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA).

Feeding experiment with ^{59}Fe

At 12 DAT, plant roots were washed with deionized water and transferred to feeding solutions with four P levels where ^{59}Fe as $^{59}FeCl_3$ (10 μmol L^{-1}) was added with or without PS (10 μmol L^{-1}), and the plants were fed for 4 h in the afternoon in beakers wrapped with aluminum foil containing 100 mL of feeding solution. Previously it has been shown that PS is not released during 4 h treatments in the afternoon (Kawai *et al*. 1988). The starting time of ^{59}Fe feeding was 8 h after the onset of light in the phytotron. The radioactivity of ^{59}Fe in each beaker was 37 kBq. The apoplastic ^{59}Fe in the roots was solubilized and removed after feeding using the method of Bienfait *et al*. (1985). After that, the plants were thoroughly washed with tap water, divided into shoots and roots, oven-dried at 70°C for 1 day and weighed.

Measurement of ^{59}Fe

Dried shoots and roots of the plants were digested in concentrated nitrate as described by Zarcinas *et al*. (1987). The radioactivity of ^{59}Fe in the digested plant solutions or the root washings containing apoplastic ^{59}Fe was determined using a gamma scintillation counter (Auto Well Gamma System, AccuFLEX ARC-7000, Aloka, Tokyo, Japan). The amount of the extracellular ^{59}Fe in the root apoplast was not included in the ^{59}Fe content in roots. The total absorption represents the sum of the amount of ^{59}Fe in the shoots and roots, and the absorption activity per root dry weight (DW) represents the total amount of ^{59}Fe in the plant divided by the root dry weight. The translocation per plant represents the shoot content of ^{59}Fe, and the translocation per shoot DW was also calculated. The relative translocation rate represents the percentage of the ^{59}Fe translocated from roots to shoot to the total absorption of ^{59}Fe per plant.

Statistical analysis

The experiment was arranged in a completely randomized block design with three replicates. Data were

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Table 1 Dry weight and chlorophyll index in barley plants grown in Fe-deficient media with different P levels at 12 days after treatment

Treatment P (μmol L^{-1})	500	50	5	0.5
Shoot DW (mg $bunch^{-1}$)	467 ± 45.9 c	812 ± 44.5 a	669 ± 32.4 ab	576 ± 12.8 b
Root DW (mg $bunch^{-1}$)	213 ± 9.0 b	418 ± 8.3 a	348 ± 21.4 a	329 ± 20.4 a
Chlorophyll index (SPAD value)	3 ± 0.58 b	23 ± 1.9 a	24 ± 1.6 a	26 ± 1.8 a

Means with standard deviation followed by different letters in each column are significantly different ($P = 0.05$) according to Duncan's Multiple Range Tests. DW, dry weight.

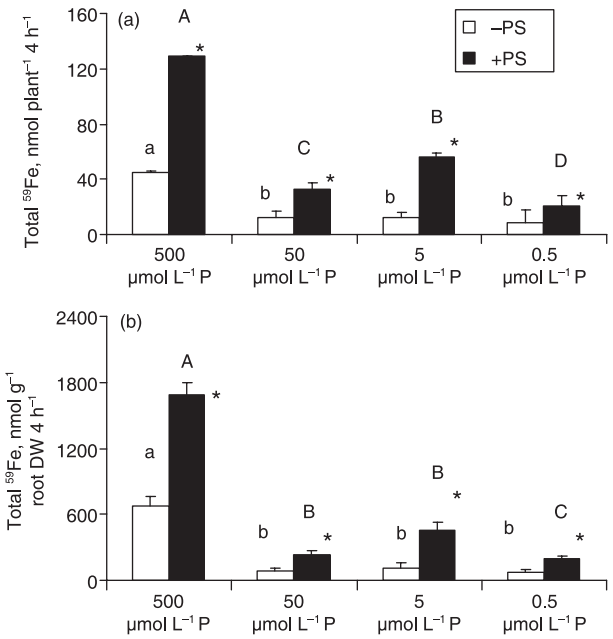

Figure 1 (a) Total absorption and (b) root absorption activity of ^{59}Fe in barley plants grown in Fe-deficient media with different P levels, with or without phytosiderophores (PS) (small letters for plants without PS and capital letters for plants with PS). Different letters indicate significant differences ($P < 0.05$) among P levels. Asterisks indicate significant differences ($P < 0.05$) between treatments with or without PS at each P level. DW, dry weight.

subjected to an ANOVA (SAS Institute 1988). The treatment means were compared by Duncan's Multiple Range Test ($P < 0.05$) using the computer "Origin 5" at Iwate University.

RESULTS AND DISCUSSION

Dry weight and chlorophyll index of the plants

The dry weights of the shoots and roots and the chlorophyll index were higher in plants grown under low P (50, 5 and 0.5 μmol L^{-1} P) and –Fe conditions compared with those grown in high P (500 μmol L^{-1} P) medium (Table 1). Consistent with a previous report, we found that the leaves of low P grown plants were green, while those of high P grown plants showed Fe chlorosis (Ladouceur *et al.* 2006).

Total absorption and root absorption activity of ^{59}Fe in plants

The total absorption of ^{59}Fe was found to be the highest in high P (500 μmol L^{-1} P) conditions even in the presence or absence of PS (Fig. 1a). In contrast, the total absorption of ^{59}Fe was reduced in low P (50, 5, and 0.5 μmol L^{-1} P) conditions. Phytosiderophores enhanced the absorption of ^{59}Fe in all conditions. In the –PS condition, the total absorption of ^{59}Fe was similar in plants grown in low P (50, 5, and 0.5 μmol L^{-1} P) media. The addition of PS to the feeding solution increased the total absorption of ^{59}Fe in plants by 1.3–3.7-fold regardless of the P level of the medium. The absorption activity of ^{59}Fe in plants per gram root DW showed a similar pattern (Fig. 1b). It was clear that plants grown in low P showed a lower Fe uptake activity compared with plants grown in high P, despite the larger root system and greener leaves of the former (Table 1). We also found that low P inhibits the ^{59}Fe absorption activity of plants irrespective of the presence or absence of PS in the medium. Previously, it has been shown that Fe chlorotic plants with high P concentrations in the shoot and roots had lower Fe content in the shoots than non-chlorotic plants with low P concentrations in the shoot and roots (Ladouceur *et al.* 2006). This higher absorption activity of ^{59}Fe in the high P condition may result from a higher response to Fe deficiency in plants. Plants grown in –Fe and high P medium may express chlorosis and show higher absorption activity of Fe. The activity of the transporter for Fe in the root plasma membrane of plants might be enhanced by –Fe and high P.

The addition of PS to the medium enhanced both the total ^{59}Fe absorption per plant and the ^{59}Fe absorption activity per root DW (Fig. 1a,b) in all P levels. The release of phytosiderophores is dependent on ATP (Takagi 1990). The ATP concentration in plant roots might be reduced by a low P level in the medium. Studies

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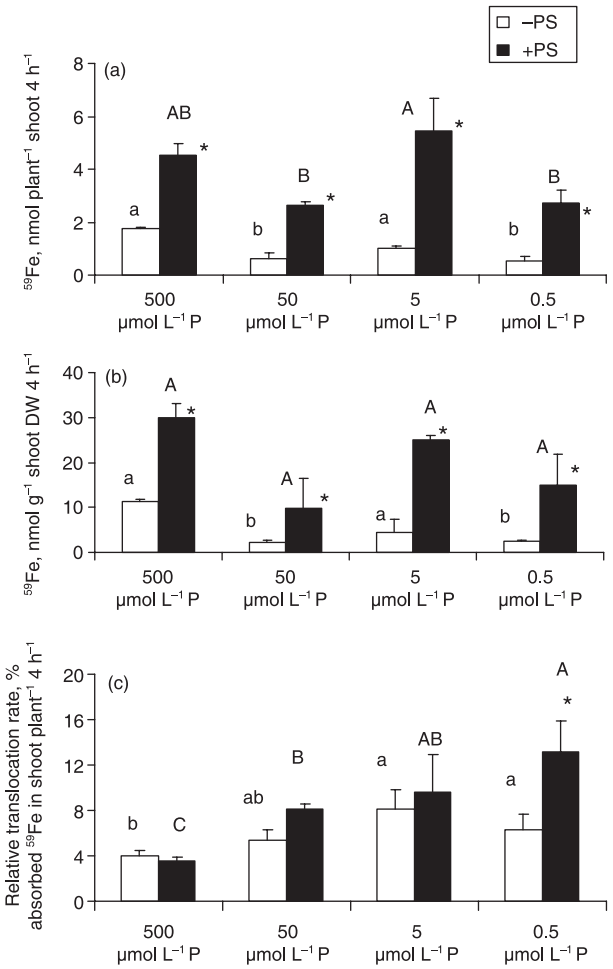

Figure 2 (a) Translocation, (b) concentration in shoots and (c) relative translocation rate of ^{59}Fe to shoots in barley plants grown in Fe-deficient media with different P levels, with or without phytosiderophores (PS) (small letters for plants without PS and capital letters for plants with PS). DW, dry weight.

have shown that low P plants can release PS (Ladouceur *et al.* 2006); thus, ATP consumption for PS release might not be critical. It was noticeable that enhancement of the absorption of ^{59}Fe by PS was found even under critical P-deficient conditions. In this experiment, although PS was equally fed to roots grown in high or low P conditions, ^{59}Fe absorption by low P plants was still lower compared with that of plants grown in high P. The ATP concentration in roots may affect the absorption of the PS–^{59}Fe complex. Further experiments are required to elucidate the relationship between PS–Fe absorption and ATP concentration in roots.

Translocation and the relative translocation rates of ^{59}Fe to shoots

The translocation of ^{59}Fe to the shoots in the –PS condition was not affected by low P levels (Fig. 2a). The addition of PS significantly enhanced ^{59}Fe translocation in each P level (Fig. 2a). Translocation of ^{59}Fe was enhanced 1.5-fold in high P (500 μmol L^{-1} P) plants and 3.2–4.3-fold in low P (50, 5 and 0.5 μmol L^{-1} P) plants. The concentration of ^{59}Fe in shoots followed the trend of ^{59}Fe translocation in shoots (Fig. 2a,b). High P concentration in plant tissues may hamper Fe translocation to leaves (Hue and Nakamura 1988; Jones *et al.* 1972). Consistent with this result, we found that high P in plants decreased Fe mobilization in roots and, thus, decreased the rate of ^{59}Fe translocation to the shoots.

Phytosiderophores-induced enhancement of ^{59}Fe translocation was observed in all P levels. However, the translocation of ^{59}Fe was enhanced at a higher ratio in low P media (Fig. 2a). The PS–Fe^{3+} complex is first absorbed via a specific transporter in roots (Murata *et al.* 2006) and then translocated to other parts of the plant (Römheld and Marschner 1986; Takagi *et al.* 1984). In barley, the addition of PS in Fe-stressed medium enhanced Fe translocation into the xylem tubes (Kawai and Alam 2006). Furthermore, it has been shown that the membrane protein that mediates the absorption of the PS–Fe^{3+} complex in maize (*Zea mays* L.) and the levels of ys1 mRNA (the gene encoding the membrane protein directly involved in the uptake of PS–Fe^{3+}) increased in both shoots and roots under Fe-deficient conditions (Curie *et al.* 2001). The activity for Fe translocation in plants at the loading site around the xylem tube may not be affected much by low P (50, 5 and 0.5 μmol L^{-1} P) conditions of the medium.

The relative translocation rate of ^{59}Fe was higher in the low P (50, 5 and 0.5 μmol L^{-1} P) plants than in the high P (500 μmol L^{-1} P) plants with or without PS (Fig. 2c). It appeared that the high P condition depressed the relative translocation rate of ^{59}Fe to the shoots. This reduced relative translocation rate of ^{59}Fe to shoots may result from the immobilization of ^{59}Fe in the form of phosphate–iron complexes in root cells (Oh *et al.* 1996). Furthermore, it has also been shown that Fe forms insoluble oxides, makes complexes with phosphates and phytoferritin in older leaves and other plant parts, which also immobilize Fe (Oh *et al.* 1996). The immobilization of Fe may subsequently diminish its movement into the phloem for long-distance translocation (Taiz and Zeiger 2002). The relatively low translocation of Fe under high P (500 μmol L^{-1} P) conditions may result in less availability of Fe in the sites of chlorophyll synthesis in leaves and subsequently induce leaf chlorosis. In contrast, the higher relative translocation rate of Fe in low P (50, 5 and 0.5 μmol L^{-1} P) conditions possibly enhances the supply of Fe to the sites of chlorophyll synthesis in leaves and reverts the leaf chlorosis phenotype.

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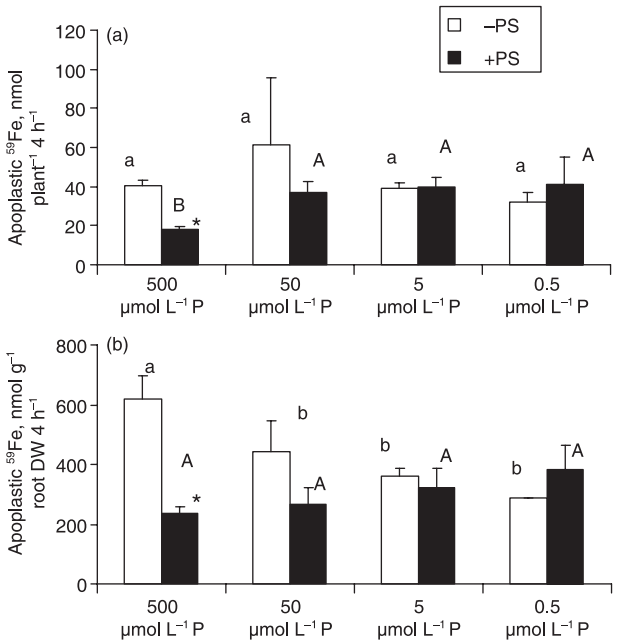

Figure 3 (a) Total absorption and (b) root absorption activity of apoplastic ^{59}Fe in roots in barley plants grown in Fe-deficient media with different P levels, with or without phytosiderophores (PS) (small letters for plants without PS and capital letters for plants with PS). DW, dry weight.

The effect of PS on the ^{59}Fe relative translocation rate was observed only at the lowest P (0.5 µmol L^{-1} P) concentration (Fig. 2c), suggesting that PS is more effective under severe P-deficient conditions. However, the physiological mechanism of this phenomenon is still unclear and needs to be further examined.

Apoplastic ^{59}Fe in roots

The root apoplastic ^{59}Fe per plant was not significantly affected by P levels in the –PS condition (Fig. 3a). In the presence of PS, the apoplastic ^{59}Fe per plant was higher in the low P (50, 5 and 0.5 µmol L^{-1} P) plants and lower in the high P (500 µmol L^{-1} P) plants (Fig. 3a). We speculate that PS depressed the formation of apoplastic Fe–phosphate complexes in high P plants, probably through solubilization of Fe in the media, preventing adsorption of Fe to the cell wall or membrane. In the presence of high P (500 µmol L^{-1} P), apoplastic Fe forms an insoluble Fe–phosphate complex (Oh *et al.* 1996) and the presence of PS may depress the formation of such an Fe–phosphate complex. It has been shown that PS does not affect this parameter in low P media, but does decrease it in a high P (500 µmol L^{-1} P) medium.

The apoplastic ^{59}Fe per root DW of the plants without PS was lower in low P (50, 5 and 0.5 µmol L^{-1} P) media than in the high P (500 µmol L^{-1} P) medium (Fig. 3b). In the presence of PS, the concentration of apoplastic ^{59}Fe was not significantly affected by the P level of the medium.

Without PS feeding, the ratio of the ^{59}Fe content in root apoplast (Fig. 3a) to the total absorption of ^{59}Fe per plant (Fig. 1a) was 0.9 in the high P (500 µmol L^{-1} P) condition, but was 5.2, 3.3 and 3.9 in the low P (50, 5 and 0.5 µmol L^{-1} P) conditions, respectively. With PS feeding, the ratio was 0.14 in the high P (500 µmol L^{-1} P) condition, while in the low P (50, 5 and 0.5 µmol L^{-1} P) conditions these values were 1.1, 0.7 and 2.0, respectively. In general, the ratio was higher in the –PS condition, suggesting that PS enhanced Fe absorption and prevented the formation of an apoplastic Fe complex containing phosphate in this short-term experiment. In addition, the ratio was much lower in the high P condition. We assume that there is a balance between the absorption and accumulation of apoplastic Fe. It appears that plants with a higher Fe demand, such as the plants in the 500 µmol L^{-1} P treatment, induce a lower ratio, indicating that Fe was preferentially absorbed rather than accumulated in apoplast. The mechanism of the formation of apoplastic Fe has not been clearly elucidated, and the function of apoplastic Fe has not been discussed sufficiently, other than playing a role as a reservoir of Fe in roots of wheat (Zhang *et al.* 1991). In soybeans, it has been shown that the accumulation of short-term Fe reserves in the root apoplast and the translocation of Fe in large quantities to the shoot may play an important role in making some cultivars resistant to Fe-deficiency induced chlorosis (Longnecker and Welch 1990). In addition to the ratio of apoplastic Fe and total absorption of Fe, a low relative translocation rate in high P (500 µmol L^{-1} P) plants was shown (Fig. 2c). This indicated that the high P condition immobilized more Fe in the roots. It will be interesting to investigate the spatial and chemical characteristics of the repression of Fe translocation in high P plant roots. Further investigation about the mechanism of the immobilization of Fe by P is necessary.

ACKNOWLEDGMENTS

A. Ladouceur thanks the Ministry of Education, Science and Culture of the Japanese Government for providing him with a scholarship that made possible this study. Thanks are due to Dr MD. Abul Kashem and Dr Abidur Rahman for their assistance with this manuscript.

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