Title

High nitrogen deposition may enhance growth of the new hybrid larch F_1 growing at two phosphorus levels

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Abstract

The recently developed hybrid larch F_1 (F_1: *Larix gmelinii* var. *japonica* × *L. kaempferi*) is being planted widely in re- and afforestation projects in northeast Asia. Nitrogen (N) deposition to forest ecosystems has been rapidly increasing in this region, due mainly to industrialization and overuse of N-fertilizer. Together with excess N, phosphorus (P) is considered to be the key determinant of tree growth in northeast Asia, because most soils have originated from immature volcanic ash. To predict the response of the F_1 to increasing N deposition and its relation with soil P availability related to immature volcanic ash soil in northern Japan, planting stocks of F_1 were grown in potted brown forest soil and categorised into eight treatments, comprising four N treatments, which covers the amount of N deposition observed and predicted in northeast Asia, in combination with two P levels. The N application increased the biomass and the light saturated net photosynthetic rate (A_{sat}) of the F_1 at all concentrations. Irrespective of expectation, P did not have any effect on these parameters. As N application increased the content of potassium (K), magnesium (Mg) and chlorophyll (Chl) in needles, a positive correlation was found between the content of N, P, K and A_{sat}. These results suggest that N deposition improves the growth of the hybrid larch F_1 at least by improving the needle N condition, as well as the concentration of other macro nutrients in the initial stage of plantation.

Key words:

Larix gemelinii × L. kaempferi,nitrogen deposition, phosphorus limitation, photosynthesis

Introduction

Larch species are recognized as important afforestation species, and have been planted intensively in a large area in northern hemisphere (Matyssek and Schulze 1987a, b; Zhang et al. 2000; Kayama et al. 2009; Mao et al. 2010). The Japanese larch (*Larix kaempferi*) is a key afforestation species that has been widely planted in northern hemisphere, especially in whole Hokkaido Island except high mountain range (Koike 2009; Ryu et al. 2009). Unfortunately, it has high susceptibility to biotic and abiotic stresses (Koike et al. 2000). To avoid these problems, the hybrid larch F_1 (*L. gmelinii* var. *japonica* × *L. kaempferi*) was recently developed by crossing female Dahurian larch (*L. gmelinii* var. *japonica*) with the Japanese larch; it is now being planted in northeast Asia in fertile soil (Kita et al. 2009; Ryu et al. 2009). However changing environment is greatly progressing (e.g. Watanabe et al. 2006).

Nitrogen (N) is an essential element for plant photosynthesis and growth (e.g. Evans 1989), but Aber et al. (1998) and Ogawa et al. (2006) have suggested that N saturation has occurred recently. In parts of Asia, Europe and North America have reported adverse environmental effects of increasing N deposition (Galloway et al. 2004). In eastern Asia, nitrogen deposition has been increasing with the rapid development of industries and excessive use of N fertilizer (Galloway et al. 2004). N deposition has been reported to exceed 25 kg N ha^{-1} $year^{-1}$ in Europe (Binkley et al. 2000), and in the Netherlands N deposition exceeded 50 kg N ha^{-1} $year^{-1}$ and some regions even reached 100 kg N ha^{-1} $year^{-1}$ (Wright and Rasmussen 1998). From 2003 to 2007, 12 monitoring sites throughout Japan found that total N deposition varied from 3.1 to 18.2 kg N ha^{-1} $year^{-1}$ (Japanese Ministry of the Environment, 2009).

Conversely, phosphorus (P) deficiency can easily arise in forests by mechanisms that include depletion-driven limitation, soil-barrier, low-P parent material, and sink-driven and anthropogenic limitations (Vitousek 2010). P is an essential macronutrient for all functions including photosynthesis. Parts of some northern hemisphere forests are already suffering from P deficiency, since P had been fixed in soil and is unavailable to plants (e.g. Schulze et al. 2005; Yi et al. 2007), especially immature volcanic ash soil (Kayama et al. 2007). Aber et al. (1998) and Braun et al. (2010) suggest that P limitation, in particular needle P deficiency, may change under conditions of excess N. They do not specify any relation between abundant N and P limitation in respect of tree growth. The correlation between leaf N and A_{sat} is generally stronger in poor N conditions, but it is weaker if both N and P contents derive from poor soil in tropical regions (Reich et al. 1994). Reich et al. (1994) suggested that a weak correlation between needle N and A_{sat} may result in limitation of P or other elements. Although brown forest soil is a common soil type in Japan (comprising 40% of land according to Nakaji et al. 2002), edaphic P limitation is believed to occur in most soil, which originates from volcanic ash (Rinyacho 1983).

Based on previous studies (e.g. Linder 1987; Aber et al. 1998), we hypothesize that greater N availability in soil may increase the nitrogen content in leaves, and photosynthetic ability and growth of the hybrid larch F_1. However, extra N application presumably causes nutrient imbalance, together with a decline in growth of the F_1. During the decline phase, P application will overcome the growth limitation in the F_1.

To test this hypothesis, N and P were applied as independent factors to planting stocks of the F_1 growing in brown forest soil, typical soil type in Japan in order to know the baseline data for this new species. Growth, needle gas exchange rates, and the needle nutrient status of the F_1 seedlings were all studied, to determine the ecophysiological mechanisms by which N and P availability affect the growth of planting stocks of the F_1. Based on the results, we discuss the effect of P availability under the increasing N deposition that prevails in East Asia, especially the common edaphic condition with immature volcanic ash soil in northern Japan

Materials and methods

Soil and plant materials

Brown forest soil was collected from the *A* soil horizon in Sapporo Experimental Forest, which is maintained by Hokkaido University in northern Japan (43.07°N, 141.38°E, 15 m a.s.l.). The average air temperature was 20.2 ℃, the average relative air humidity was 74.3 %, and the average accumulated photosynthetic photon flux (PPF: converted by HOBO, Pendant Temperature/ Light Data Logger, 64K-UA-002-64, Onset, U.S.A.) was 236.1 mmol m^{-2} in the nursery during the growing season from June to September. The snow-free period is from May to early October.

On 23 May 2008, planting stocks of 3-year-old clonal seedlings of the hybrid larch F_1 were planted in 7.5 L pots and were grown for whole growing period here in order to evaluate the early stage of their growth. All the seedlings were harvested after one growing season on 20 October 2008.

The pots were set in the open air at the Experimental Nursery, with a matched tray to prevent nutrient leaching for each pot. During long rainy periods, water that had pooled in the tray was collected and used to irrigate the same pot. The average values (± standard deviation) of the height and stem base diameter of the seedlings at the beginning of the experiment were respectively 64.9 (±8.3) cm and 8.6 (±0.6) mm. This F_1 is characterized by its fast growth and good survival (Koike et al. 2000). This new species was created by crossing female Dahurian larch from the Kurile Islands or Sakhalin, Russia with male Japanese larch (Kita et al. 2009; Ryu et al. 2009).

Nitrogen and phosphorus treatments

The experiment was fully randomized. We used four levels of N treatment (0, 20, 50 and 100 kg N ha^{-1}; 0 mg, 62.8 mg, 157.0 mg and 314.0 mg N $seedling^{-1}$, referred to as N0, N20, N50 and N100), with ammonium nitrate solution (NH_4NO_3) simulating acid deposition, in combination with two levels of P treatment (0 and 50 kg P ha^{-1}; 0 mg and 157.0 mg N $seedling^{-1}$, referred to as P0 and P50) with potassium dihydrogen phosphate (KH_2PO_4) solution. N100 is believed to exceed the requirements of woody plants, based on studies in the vicinity of Tokyo metropolis, which is the most polluted part of Japan (e.g. Nakaji et al. 2002; Watanabe et al. 2006; Magnani et al. 2007; Kimura et al. 2009). At the same time, potassium chloride (KCl) was supplied to the soil that did not receive KH_2PO_4 in order to provide an equal amount of K among all treatments. There were 3 replications for each treatment. N and P were supplied to the soil three times, with one-third of total amount on 18 June, 15 July and 21August.

Soil analysis

Soil samples were taken from the potted soil at the end of the experimental period following harvesting of the seedlings. Soil samples were air-dried at room temperature for one month, prior to determination of the total C and N content using a NC analyzer (NC-900, Sumica-Shimadzu, Osaka/Kyoto, Japan). The soil pH was measured with a pH meter (M-12, Horiba, Japan) after 10 g fresh soil had been shaken for 1 h with 50 ml of ion-exchanged water. Available P was extracted by the Bray-2 method (Bray and Kurtz 1945), and determined by the molybdenium blue method with ascorbic acid (Murphy and Riley 1962), using a spectrophotometer (Gene spec III, Hitachi, Tokyo, Japan). Nitrate and ammonium ions in soil were extracted by 2M KCl and measured with an auto-analyzer (AACS-4; BL-TEC Co. Ltd., Osaka, Japan).

Measurement of growth

At the end of the experimental period, on 20 October 2009, all seedlings were harvested in order to determine the dry mass and allocation of the plant organs. Few fallen needles were collected and also added the dry mass. The harvested samples were dried in an oven at 70˚C for 1 week and were then weighed.

Gas exchange parameters

The gas exchange rates of mature needles were measured on 12-16 September using an open gas exchange system (LI-6400, Li-Cor Inc., Lincoln, NE, U.S.A.) with a LED light source (LI-6400-40). Using three seedlings per treatment, we quantified the A/C_i curve with 13 steps of external CO_2 concentration (C_a) (e.g. Farquhar and Sharkey 1982; Long and Bernacchi 2003). During the measurements, the needle temperature was maintained at 25.0 ± 1.0˚C and the PPF (Photosynthetic Photon Flux) at 1500 μmol m^{-2} s^{-1}; these conditions had been determined previously to induce the maximum photosynthetic capacity of this species (Ryu et al. 2009). The leaf-to-air vapor pressure deficit was maintained at around 1.5 kPa, corresponding to ambient water vapor conditions in the field we used. The A/C_i curve was used to estimate the net assimilation rates (A_{sat}) and stomatal conductance (g_s) at 380 μmol mol^{-1} CO_2, the net assimilation rate at 1700 μmol mol^{-1} CO_2 (A_{max}), the maximum rate of carboxylation (V_{cmax}), and the maximum rate of electron transport (J_{max}). The Rubisco Michaelis constants for CO_2 (K_c) and O_2 (K_o) and the CO_2 compensation point in the absence of dark respiration (Γ*), used in analysis of the A/C_i curve, were calculated according to the method of Bernacchi et al. (2001).

Needle nutrients

After measurement of the gas exchange rate, the needles were collected to determine the leaf (=needle) mass per unit area (LMA). The LMA was calculated based on the needle projected area, determined with an image scanner (CanoScan LiDE 600F, Canon, Tokyo, Japan), and the dry mass of the samples. For analysis of the needle nutrient status, the dried needles were ground into fine powder. The N content was determined by the combustion method using a NC analyzer. The samples were digested by HNO_3, HCl and H_2O_2, after which an inductively coupled plasma-atomic emission spectrometer (ICP-AES, IRIS/IRIS Advantage ICAP, Thermo Fisher Scientific Inc., Massachusetts, U.S.A.) was used to determine the content of N, P, K and Mg in the needles. Needle samples for chlorophyll were stored in a refrigerator at -80˚C. The chlorophyll in the needles was extracted with dimethyl sulfoxide according to the method of Barnes et al. (1992) and Shinano et al. (1996). The absorbance of the extract was measured at 664.9 nm and 648.2 nm using a spectrophotometer (Gene spec III, Hitachi, Tokyo, Japan), and the chlorophyll content was calculated according to Barnes et al. (1992).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to evaluate the effect of N and P loading, and of their combined effect, on the physiological and growth properties of seedlings of the hybrid larch F_1 and on soil properties. All tests were performed using SPSS 16.0 statistical software.

Results

Soil characteristics

Soil pH in the pots was measured after one growing season. The pH ranged from 4.94 to 5.20. It increased significantly with addition of P (Table 1). In the high N and P (P50N100) treatment in particular, the available P concentration in soil was up to 0.28 g kg^{-1}. The total N content (TN) in the soil was significantly increased by N and P loading (ranging from 3.72 g kg^{-1} to 4.66 g kg^{-1}). The soil C/N ratio decreased significantly (ranging from 13.68 to 11.20) with N and P application; there was no interaction between N and P application.

Growth

The needle dry mass was significantly increased by N loading (Fig. 1); the needle biomass of P0N100 was 60.58% greater than in P0N0. The dry mass of stem and roots also increased significantly with N loading. No significant effect of P loading was observed on biomass parameters, nor any significant interaction of N and P loading.

Needle gas exchange parameters

N application caused the photosynthetic parameters to increase significantly. Increase of the N supply from 0 to 100 kg N ha^{-1} led to increases in A_{sat}, g_s, V_{cmax}, J_{max} and A_{max} (Table 3). In contrast, P application had no significant effect on any of these parameters. There were significant increases in the P and chlorophyll content with P application (Table 2). We found a positive correlation of A_{sat} with the content of N, of K and of Mg in needles (Fig. 2). No significant effect of N and P application on LMA was observed (Table 3).

Needle nutrients

The needle N concentration was increased by N loading, and varied from 1.16 g m^{-2} to 1.62 g m^{-2} (Table 2). Furthermore, the needle P concentration (varying from 33.02 mg m^{-2} to 46.27mg m^{-2}) increased significantly with P application. Needle K also increased significantly with N application (it ranged from 18.28 mg m^{-2} to 3.88 mg m^{-2}). Needle Mg increased significantly from 4.95 mg m^{-2} to 10.16 mg m^{-2} with N loading. The needle C/N ratio decreased significantly with N application (from 41.08 to 28.09 g g^{-1}), and there was no large difference in the LMA with the N and P treatments. The concentration of chlorophyll (Chl), increased significantly with N and P application (it ranged from 109.67 mg m^{-2} to 176.65 mg m^{-2}).

Discussion

Effects of nitrogen and phosphorus loading on needle gas exchange parameters

Although we are suffering from high N deposition (Izuta 2006), we should find the way for sustainable forest management as well as produce forest resources (Yi et al. 2007). In northeast Asia, many efforts are provided for several kinds of tree species, such as Korean pine (Yi et al. 2007), Red pine (Choi 2008), Sakhalin spruce (Kayama et al. 2007), Japanese larch (Kayama et al. 2009), etc. These researches provide essential relationship between N and P concentration in needles accompanied by the role of symbiotic micro-organisms (Choi 2008; Kayama et al. 2009). N treatments increased the growth of the hybrid larch F_1 over the whole range of N applications. These results suggest that N continued to be the limiting factor on the growth of the F_1 in our experiment. N application increased the photosynthetic parameters together with the chlorophyll, Mg and K content in needles. N application would usually reduce the soil pH (e.g. Schulze et al. 2005; Marschner 1995); when the pH is below 7, soil P will be found in the soil solution mainly as $H_2PO_4^-$ or HPO_4^{2-}, which can generate phosphate with K^+ and Mg^{2+}

cations (Schulze et al. 2005; BassiriRad 2005) . No fall in pH was observed in the present study with N addition, probably because of the large buffering capacity of brown forest soil.

The photosynthetic rate A_{sat} is correlated significantly with leaf N (Matyssek and Schulze 1987a; Evans 1989), and also with leaf Mg. Leaf Mg is essential components of chlorophyll, and are usually positively correlated with the photosynthetic rate (e.g. Marschner 1995; Lambers et al. 2008; Schulze et al. 2005). Needle K and Mg are also used in adjusting the osmotic potential of cells, and stabilizing the pH in needles (Wu and Berkowitz 1992; Yi et al. 2007). These results imply that N deposition in soil cannot by itself give rise to the nutrient imbalance in needles given the uptake of other macro-nutrients by seedlings, or the resulting increase in growth. As photosynthetic production in larch is strong depending on the arrangement of needles in a crown (Matyssek and Schulze 1987b), N application leads to greater photosynthetic capacity in the F_1, which induces an increase in the whole-plant biomass. This increase may give rise to the enzyme activity which may helpful of transporting more nutrients, especially K and Mg, which are limiting factors for photosynthesis, thereby allowing further improvement in growth by utilizing N uptake by the roots of the tree (Qu et al. 2004; Kayama et al. 2009).

Stomatal conductance increased with N application (Table 3), which may increase the photosynthetic rate (e.g. Matyssek and Schulze 1987a; Evans 1989). We can find no reason why; while A_{sat} and needle P concentration showed significant positive correlation, P application did not enhance the photosynthetic parameters or growth of the F_1. To understand how N deposition affects these trees, the dynamics of further nutrients must evidently be studied.

Afforestation by Hybrid larch F_1

Recently many trails are carried out for forest regeneration and rehabilitation with conifers in degraded area in northeastern part of Asia (Yi et al. 2007; Choi 2008; Kayama et al. 2007; 2009; Ryu et al. 2009). They try to use endemic tree species in practical forestry and restoration for keeping biodiversity. However, we also examine to use hybrid larch F_1 as for increasing wood stock for restricted area for commercial forestry, too (Kita et al. 2009). For this objective, we try to study on further possibility to use F_1 as a promising planting stock (Kita 2011).

Regardless of our hypothesis that extra N application presumably causes nutrient imbalance, together with a decline in growth of the F_1, we did not find any decline in the F_1 due to N saturation, even with an extreme amount of N loading of the soil (Izuta 2006). However, P loading did not have significant effects on the hybrid larch F_1. It is possibly because of the soil already included enough phosphate; the P effects were not shown significantly though we supplied P (Table 1). Another possible reason is that the hybrid larch F_1 needs less P than N, however, this speculation needs to be tested. Interestingly, our previous study found that the growth of *L. kaempferi* (a pollen parent to the F_1) improved with N loading in brown forest soil (Watanabe et al. 2006). In addition, Makoto et al. (2010) found that P, rather than N, is the most important factor determining the growth of *L. gmelinii* (a mother tree to the F_1) seedlings, but the F_1 is sensitive to N in brown forest soil. Extensive meta-analysis shows that the N utilization capacity is highly species-specific (e.g. Aerts and Chapin 2000), and the relation of the nutrient economy in the F_1 trees to that in its parents is little understood (e.g. Ryu et al. 2009).

A loading of 100 kg N ha^{-1} is believed to be far beyond the requirements of plants, simulating the maximum value predicted around Tokyo, the most polluted region in Japan (Nakaji et al. 2002, Kimura et al. 2009). Recently, we found severe nutrient imbalance in the planting stock of F_1 planted in serpentine soil treated with a simulated acid rain of ammonium sulfate for 3 years (Watanabe et al. 2012). These results suggest that except serpentine soil the F_1 is a superior species for afforestation than the Japanese larch, not only because of its resistance to drought and frost stress (Ryu et al. 2009), but also because of its strong preference for N loading to soil, which is increasing in Asian countries.

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Table 1. Soil characteristics for four levels of N application (0, 20, 50 and 100 kg N ha^{-1}) and two levels of P (0 and 50 kg P ha^{-1}).

	pH	C/N （g g^{-1}）	TN (g kg^{-1})	avaliable-P (g kg^{-1})
P0N0	4.94 (0.13)	13.68 (0.19)	3.72 (1.90)	0.15 (0.07)
P0N20	5.09 (0.07)	13.64 (0.08)	3.89 (0.82)	0.18 (0.02)
P0N50	5.04 (0.12)	12.50 (0.79)	4.24 (7.86)	0.13 (0.07)
P0N100	5.03 (0.13)	12.03 (0.62)	4.48 (6.20)	0.14 (0.08)
P50N0	5.14 (0.10)	11.87 (0.19)	4.06 (1.88)	0.20 (0.16)
P50N20	5.08 (0.08)	11.63 (0.57)	4.49 (5.74)	0.22 (0.13)
P50N50	5.10 (0.09)	11.99 (1.13)	4.21 (11.29)	0.17 (0.04)
P50N100	5.20 (0.03)	11.20 (0.88)	4.66 (8.83)	0.28 (0.05)
N	n.s.	*	**	n.s.
P	*	***	*	***
N×P	n.s.	n.s.	n.s.	n.s.

Data are average values (standard deviation) calculated from soil samples; the four levels of N (0, 20, 50 and 100 kg N ha-1) are denoted N0, N20, N50 and N100; the treatments with P (0 and 50 kg P ha-1) are denoted P0 and P50; ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant

Table 2. Needle nutrients, C/N ratio, chlorophyll (Chl.) content and LMA of the hybrid larch F_1 grown with application of the four N levels and two P levels.

	N(g m^{-2})	P(mg m^{-2})	K(mg m^{-2})	Mg(mg m^{-2})	C/N(g g^{-1})	Chl. (mg m^{-2})	LMA (g m^{-2})
P0N0	1.16 (0.22)	36.43 (7.32)	21.26 (9.79)	4.95 (1.24)	41.08 (3.75)	109.67 (21.72)	90.70 (11.05)
P0N20	1.22 (0.41)	33.02 (12.32)	18.28 (4.06)	6.13 (1.30)	37.27 (2.65)	109.85 (7.33)	75.82 (2.30)
P0N50	1.21 (0.32)	38.72 (2.11)	27.85 (1.80)	6.11 (0.66)	35.47 (6.62)	128.87 (35.54)	82.97 (14.58)
P0N100	1.62 (0.14)	35.98 (3.31)	29.78 (9.03)	9.42 (3.56)	30.09 (3.81)	161.87 (20.91)	94.54 (4.53)
P50N0	1.26 (0.11)	45.87 (9.34)	21.07 (2.97)	5.86 (0.93)	37.74 (1.17)	137.24 (14.30)	93.33 (10.58)
P50N20	1.15 (0.13)	46.27 (12.85)	21.96 (3.87)	6.10 (0.45)	36.84 (0.88)	133.68 (12.04)	84.08 (10.21)
P50N50	1.25 (0.15)	42.14 (1.76)	25.69 (12.17)	7.85 (3.96)	33.81 (6.91)	155.61 (20.14)	82.66 (7.29)
P50N100	1.38 (0.39)	38.15 (3.99)	34.88 (8.74)	10.16 (2.02)	28.09 (1.89)	176.65 (33.45)	90.72 (2.88)
N	**	n.s.	*	*	**	**	n.s.
P	n.s.	*	n.s.	n.s.	n.s.	*	n.s.
N×P	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Data are average values (standard deviation); the four levels of N (0, 20, 50 and 100 kg N ha-1) are denoted N0, N20, N50 and N100; the treatments with P (0 and 50 kg P ha-1) are denoted P0 and P50; ANOVA: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant

Table 3 Photosynthetic parameters of hybrid larch F_1 seedlings raised under the four N levels and two P levels loading.

[redacted]

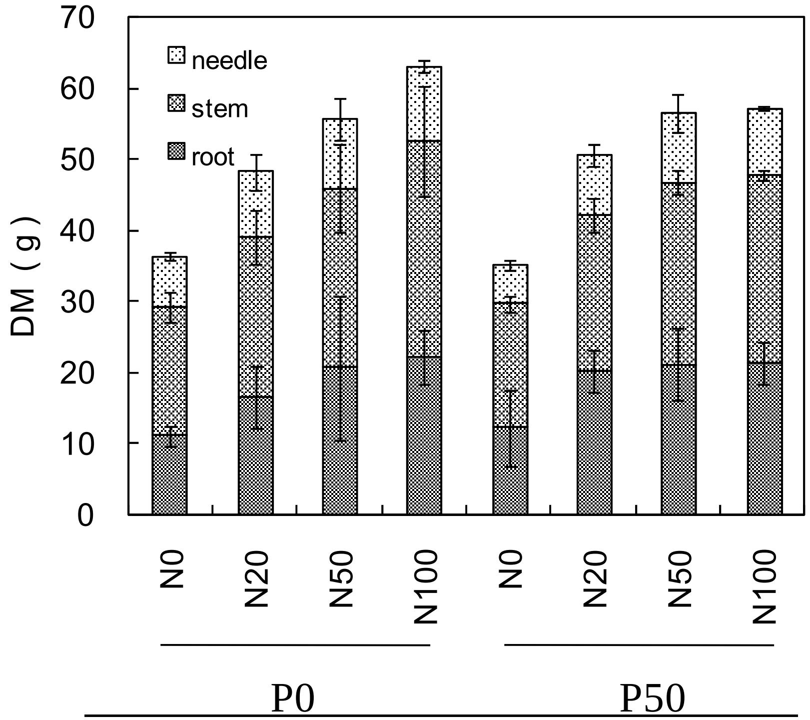
Data were the average values (standard deviation); four levels of N (0, 20, 50 and 100 kg N ha-1) are referred as N0, N20, N50 and N100 respectively; the treatments of P supply (0 and 50 kg P ha-1) referred as P0 and P50 respectively; ANOVA: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

Legends for Figures

Fig. 1 Needle, stem and root dry mass of the hybrid larch F_1 raised with application of the four N levels and two P levels. All the data are average values (standard error); four levels of N (0, 20, 50 and 100 kg N ha^{-1}) are referred as N0, N20, N50 and N100 respectively; the treatments of P supply (0 and 50 kg P ha^{-1}) referred as P0 and P50 respectively; *, P < 0.05; **; n.s., not significant

Fig. 2 Relations between light and CO_2 saturated net photosynthetic rate (A_{max}) and needle N, P, K and Mg contents of the hybrid larch F_1 treated with four N levels and two P levels. Each plot indicates the value in each individual seedling. Regression line for all treatments was obtained using the reduced major axis regression method. Open triangle stands for P0, solid cycle stands for P50.

[y = 4.15x+2.08, R^2 = 0.23 for needle N vs. A_{sat} (P < 0.001); y = 5.81x +7.162, R^2 < 0.01 for needle P vs. A_{sat}, (P = 0.042); y = 172.72x +3.06, R^2 = 0.39 for needle K vs. A_{sat}, (P < 0.043); y = 690.45x + 2.51, R^2 = 0.61 for needle Mg vs. A_{sat}, (P = 0.015)]



ANOVA	needle	stem	root
N	**	**	*
P	n.s.	n.s.	n.s.
N×P	n.s.	n.s.	n.s.

Fig. 1

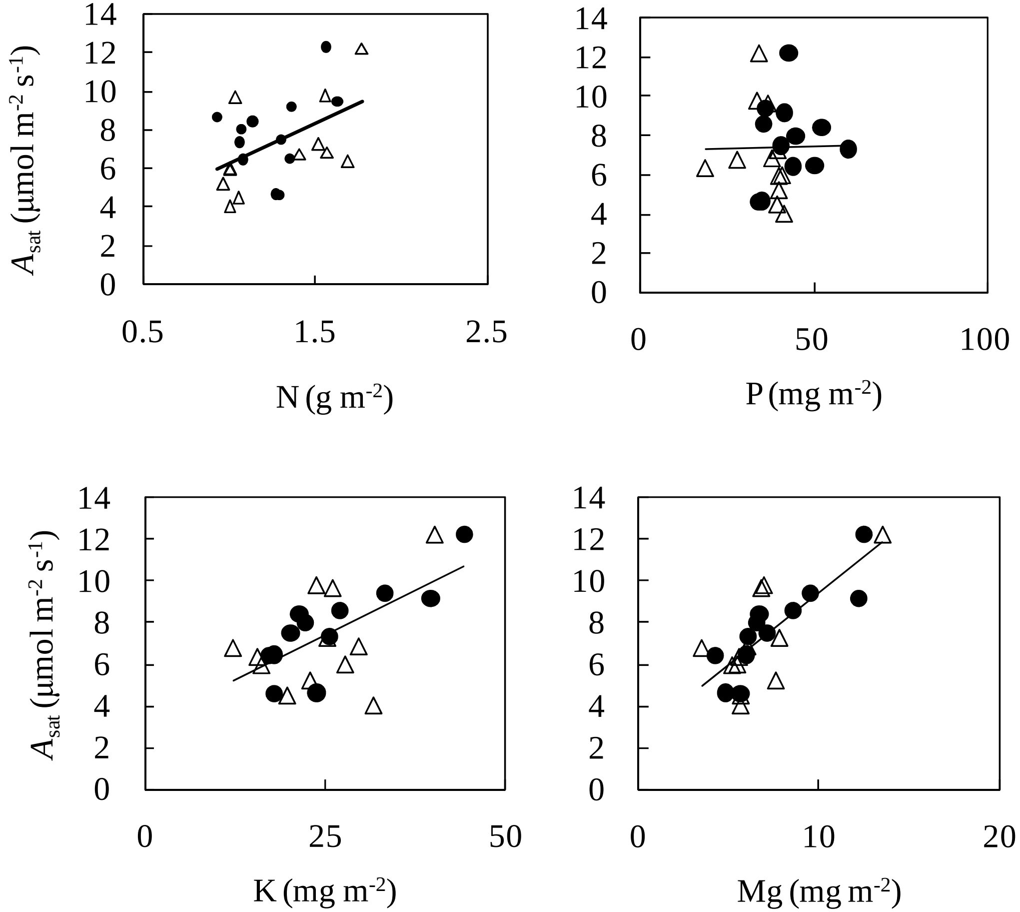


Fig. 2