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Defense characteristics of seral deciduous broad-leaved tree seedlings grown under differing levels of CO_2 and nitrogen

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Abstract

Mixed broad-leaved forests are abundantly populated by several kinds of herbivore species in northern Japan. The life of herbivores depends strongly on the quality of food leaves. Leaf quality is changing with increasing atmospheric CO_2 and nitrogen deposition. Four seral species of deciduous broad-leaved tree seedlings (alder, birch, oak and maple) were raised in all four combinations of two levels of CO_2 and two levels of nutrient, to examine foliar defense traits. To evaluate the relative defense capacity of the four tree species, we used wild silkworms (Erisan; a generalist herbivore) as a bioassay material. Except with alder, the survival rate and longevity (ML_{50}) of the silkworms were least when they were fed with leaves of seedlings raised under high CO_2 and infertile soil conditions, and longest on a diet of leaves grown in ambient CO_2 and fertile soil, especially in birch and maple. The longevity of Erisan decreased in the order birch, oak and maple. The longevity of Erisan fed with alder leaves was independent of CO_2 levels and was longer on alder seedlings grown in infertile soil conditions. Alder is an actinorrhizal plant that can fix atmospheric nitrogen in root nodules formed by the actinomycetes *Frankia* sp. The activity of symbiotic microbes would have been enhanced by the greater amount of photosynthates received from the host plants at high CO_2, improving the food quality for the silkworms. All but alder, leaf chemical traits especially C/N ratio were concluded to affect ML_{50} of Erisan larvae.

Key words: elevated CO_2, nitrogen deposition, seral deciduous broadleaved trees, defense chemicals, soil fertility, silkworm

Introduction

The availability of carbon and nutrient affects plant leaf chemistry, which in turn influences the activities of herbivores. Several studies have reported that defense traits of leaves are changing both physically and chemically with increasing atmospheric CO_2 concentrations and nitrogen deposition (Lincoln et al. 1993, Bazzaz 1996, Lindroth 1996, Koike et al. 2003).

Although forests are responsible for about two thirds of global photosynthetic production, few studies have focused on changes in tree chemistry and the resulting interactions with herbivores (Bazzaz 1996, Lindroth 1996, Hartley and Jones 1997, Norby and Luo 2004). The proportion of grazing by herbivores in a forest was estimated to be 4-6% during the 1960s (Furuno 1974), and 10-20% during the late 1990s (Crawley 1997). During these three decades, marked environmental changes took place: an increase in atmospheric CO_2 and acid deposition in forest ecosystems (Shibata et al. 2001, Izuta and Nakaji 2003). Forest trees will have responded to these environmental modifications. How will these changes affect grazing damage to forests caused by insect herbivores?

Several kinds of herbivore are abundant in mixed broad-leaved forests in northern Japan (Koike et al. 2003). In mixed forests, leaves of early successional species are usually grazed by herbivores while those of late successional species are rarely eaten, except by specialists such as the Beech caterpillar (*Syntypistis punctatella*) (Kamata 1999, 2000) and leaf beetles (*Chrysomela populi, Agelastica coerulea*) (Hayashi et al. 1985, Bauer et al. 1991) on species including poplar and birch (*Populus mamimowiczii, P. sieboldii, Betula platyphylla* etc.). Leaf longevity of late successional species usually exceeds that of early successional species (Mooney and Gulmon 1982, Koike 1988). It therefore seems that the defenses of late successional species are stronger. Except among introduced tree species, such as larch (*Larix kaempferi*), outbreaks of herbivores occur in mixed forests at intervals of several years (e.g. Kamata 2000). This interval may be related to tree leaf defense, including induced defense, and its duration (Jones and Coleman 1991, Kamata 1996, Haukioja and Honkenen 1997, Pickett et al. 1999, Karban and Baldwin 2001, Nabeshima et al. 2001). We should therefore examine the defense traits of woody species, in particular the timing of leaf emergence, leaf longevity, and chemical defense capacity as well as types of insect herbivores.

Survival rate and longevity differ between insect herbivores species fed with different tree species grown under the combination of high CO_2 and high nitrate nitrogen levels (Lindroth 1996, Docherty et al. 1997). Apart from specialists, longevity of insect herbivores tends to be less when they are fed with leaves at high CO_2 concentrations, especially maple (Matsuki 2003). In general, the chemicals that enhances the defense capacity of leaves increases in plants growing in infertile soil conditions (Bryant et al. 1983, Coley et al. 1985).

The defense capacity of broadleaf trees is usually considered to increase at elevated CO_2 conditions (Linclon et al. 1993, Lindroth 1996, Koike et al. 2003). The defense capacity of leaves of deciduous broad-leaved trees in infertile soil should therefore increase under increasing CO_2. However, soil fertility is also liable to change in future because of an increase in atmospheric nitrogen deposition in forests; this usually acts as a fertilizer. What are the combined effects of elevated CO_2 and soil fertility on the defense capacity of seedlings of typical tree species in northern Japan, where the soil is characterized by volcanic ash?

To answer this question, we studied the defense capacity of four types of seral deciduous broad-leaved tree seedlings raised under elevated CO_2 and ambient CO_2 in combination with two nutrient regimes. For the feeding experiment, we used a known generalist (a wild silk worm) in bioassay to determine the relative defense capacities of the tree seedlings against herbivores.

Materials and Methods

Plant materials

Individual two-year-old seedlings of mountain alder (*Alnus hirsuta*), white birch (*Betula platyphylla* var. *japonica*); early successional species, oak (*Quercus monglica* var. *crisupla*); mid-late successional species and maple (*Acer mono*); late successional species were planted in 5 liter peat pots filled with Kanuma pumice soil and clay soil (2:1 in volume), simulating infertile volcanic ash soil. Alder is a N_2-fixing plant that forms a symbiotic association in roots with an actinomycete of the genus *Frankia* sp. Four plants each were treated with factorial combination of two levels of CO_2 and soil fertilities. The nutrient condition of seedlings was regulated by the application frequency of liquid fertilizer solution (Nitrogen 140mg, N:P:K = 4:3:2, Hyponex, U.S.A.); fertile (+N) and infertile (-N) conditions were defined by supplying fertilizer solution once a week (+N) or once a month (-N). Six rooms were used for CO_2 regulation (CO_2 concentration expressed as partial pressure of CO_2 ($PaCO_2$); 72 $PaCO_2$ vs. 36 $PaCO_2$, three of each) in the phytotron belonging to the Forestry and Forest Products Research Institute in Sapporo, Japan. For the bioassay we used leaves at age 25~35 days.

Measurements

Leaf color (chlorophyll) was detected with a chlorophyll meter (SPAD-502, Minolta, Tokyo), which stands for photosynthetic activities in the same species (Koike 1990). After determination of the chlorophyll content, the leaves were weighed to find their fresh mass and then oven dried at 60 ^{o}C for 48 hrs to determine the specific leaf mass (leaf mass per unit area; LMA). LMA is one physical defense indicator of a leaf (Matsuki 2003, Matsuki et al. 2004).

The nitrogen content was measured from the dried materials using a NC analyzer (NC-900, Shimadzu, Kyoto, Japan). The amount of total phenolics was determined by the Folin-Ciocateus' method (Matsuki et al. 2004), and condensed tannin by the sulfuric acid method (Bate-Smith 1977). These leaves were sampled in part from the same leaves for feeding Erisan, and they were immediately frozen dried and stored (at -60^{o}C to maintain the quality of samples) until the chemical analysis. It is well known that condensed tannin inhibits digestion in insect larvae, and is therefore an indicator of plant defense (Schoonhoven et al. 1998). Finally, the root system of the alder was examined for the number of nodules produced per plant and was weighed, as an indicator of nodule activities (Koike et al. 1997, Tobita et al. 2005).

The feeding experiment used the wild silkworm "Erisan" (*Samia cynthia ricini*: generalist herbivore) fed with one species leaves treated with one treatment in one feeding chamber for evaluation of defense capacity in leaves among four species. Six Erisan individuals in each treatment (2 or 3 leaves) were used at conditions of 25 ^{o}C, relative humidity 75 % and 16 hr (light). We observed the development of the Erisan larvae at two day intervals, following Shibata et al. (2004). The survival days was expressed as median longevity (ML_{50}).

Statistical analysis

Two-way analysis of variance (ANOVA) for split-plot designs was used to compare the effect of CO_2 and N on the survival days of Erisan (ML_{50}) fed with each species (using log-transformed data), LMA (= leaf toughness), and chemical traits (C/N ratio, total phenolics, condensed tannin) for each species. One-way ANOVA was used to compare the survival days of Erisan (ML_{50}) (using log-transformed data), LMA, and chemical traits among treatments within each species, and among species within each treatment. The statistical significance of differences between species in these parameters at the same treatment and between treatments within same species was tested using the Tukey-Kramer post hoc test at the probability level $\alpha = 0.05$, implemented with Stat-View Version 5.0 (SAS Institute 1998) and JMP (SAS Institute 2003). The different alphabet in each figure means statistical significance.

Results

Change in longevity and survival rate of larvae

The survival curve of wild silkworm (Erisan) varied with the “food” leaves grown under different environmental conditions (Fig. 1). The survival rate of larvae fed with alder and birch was higher than with oak and maple. Independent of the CO_2 levels, the survival curve with alder fell sharply at around 15 days after the feeding treatment, whereas the curve with birch in fertile soil fell gradually from 5 days after the start of feeding. At last, a few individuals of Erisan fed with high nutrient leaves of birch could survive at around 40 days. The survival curve with birch from infertile soil fell rapidly at 3~4 days after the treatment, likely to oak and maple at all treatments. This was accelerated by 72 $PaCO_2$. However, all Erisan individuals tested were dead before they became pupae. There was no difference in the pattern of survival curve of Erisan with oak and maple leaves. Erisan fed with oak and maple leaves died at around 10 days after feeding experiments started.

The longevity expressed as ML_{50} of Erisans fed with leaves of alder at all treatments and birch at +N was longer than that with oak and maple (Fig. 2, $P < 0.0001$). Erisan longevity of oak increased in response to N, not CO_2. A significant CO_2 x N interaction term in three species except oak revealed that the MD_{50} of Erisans in response to elevated CO_2 was influenced by soil N. Among all treatments for alder, ML_{50} tended to be longest with leaves at 36 $PaCO_2$-N ($P = 0.0007$), and there was no significant difference in ML_{50} among the rest three treatments. Erisan longevity of birch and maple was unresponsive to elevated CO_2 at –N, but decreased to elevated CO_2 at +N. Erisan longevity of birch increased significantly to N at both CO_2 treatments, but in maple, increased to N only at 36 $PaCO_2$ ($P<0.0001$ for birch, and $P=0.0002$).

Characteristics of leaf color, LMA and C/N ratio of seedlings

The leaf color of birch, oak and maple grown at 72 $PaCO_2$-N was pale green (SPAD value of around 30) as compared with that at 36 $PaCO_2$-N (SPAD value about 45). This coloring was less clear for leaves in three species grown under 72 $PaCO_2$+N compared with that at 72 $PaCO_2$-N. For alder, there was no difference in leaf color between different CO_2 and nutrient levels (SPAD value was about 47).

LMA (leaf mass per unit area; $g \cdot m^{-2}$) corresponds to leaf toughness of deciduous broad-leaved tree species. Oak showed the highest LMA value among species independent of treatments (Fig. 3, $P = 0.0041$ for 72 $PaCO_2$+N, and $P<0.0001$

for the other three treatments). A significant CO_2 x N interaction term in birch and oak revealed that the LMA in response to elevated CO_2 was influenced by N addition. LMA of birch and oak increased markedly to elevated CO_2 at –N, but unresponsive to elevated CO_2 at +N. LMA of birch and oak decreased N at 72 $PaCO_2$, but unresponsive to N at 36 $PaCO_2$. The largest C/N ratio was found in birch at 72 $PaCO_2$ -N (Fig. 4). Generally, the C/N ratio in infertile soil (-N) was greater that with fertile soil (+N) irrespective of CO_2 levels, especially for birch, though, in oak, only leaves at 72 $PaCO_2$-N displayed a high C/N ratio, of about 33. Unlikely alder and maple, both CO_2 and N affected C/N ratio of birch and oak with significant CO_2 x N interaction. The C/N ratio of birch and oak increased significantly to elevated CO_2 at –N, but unresponsive to elevated CO_2 at +N, in the same way as LMA. C/N of alder was about 18.5~21.5. On average, C/N of alder was slightly larger than that of the other three species at fertile (+N) condition ($P=0.0227$ for 36 $PaCO_2$ + N, and $P=0.0071$ for 72 $PaCO_2$ + N).

Change in defense chemicals

The effects of CO_2 and N on the amount of total phenolics (in short, phenolics) were different among species (Fig. 5). Phenolics of alder declined in response to elevated CO_2, not N, which was almost opposite tendency as we expected. In birch, phenolics were significantly larger in infertile condition (-N) than in fertile (+N), but not affected by CO_2. Though both CO_2 and N affected phenolics of oak and maple, there were some differences in response to CO_2 x N interaction. The phenolics of both oak and maple were responsive to elevated CO_2 at –N, but unresponsive in oak and highly responsive in maple to elevated CO_2 at +N.

The amount of condensed tannin (in short, tannin) in leaves of birch and maple was larger than that in alder and oak at each treatment (Fig. 6). The effects of CO_2 and N on the amount of tannin were different among species. Birch leaves tended to smaller value of tannin at high CO_2 irrespective of soil fertility. In birch, oak and maple, both CO_2 and N affected the amount of tannin with significant CO_2 x N interaction. The amount of tannin of these three species increased to elevated CO_2 at –N, but unresponsive to elevated CO_2 at +N, except alder. There was a large amount of tannin in alder leaves in -N conditions, irrespective of CO_2 levels. The similar tendency was found in maple that was clear at 72 PaCO_2. .

Nodule in root

Nodule formation of alder roots increased at low soil fertility, which was significantly accelerated by elevated CO_2 (data not shown; *P=0.0001*). Dry mass of nodule of alder at +N was about 6.5 g per plant at 36 or 72 PaCO_2, while that at -N was 11.5 g per plant for ambient 36 PaCO_2 and 18.3 g per plant for elevated 72 PaCO_2.

Discussion

Defense capacities in foliage organs of woody species have been influenced by the increase in atmospheric CO_2 and the amount of nitrogen input (Lincoln et al. 1993, Lindroth 1996, Koike et al. 2003). It is expected that longevity of Erisan (wild silkworm) fed with leaves at high CO_2-N may be shorter than that at ambient CO_2+N. In fact, all but alder, the longevity of Erisan fed with leaves at infertile soil condition (-N) was shorter, however, this tendency was not accelerated by elevated CO_2. This result was not found in alder.

In previous experiments on herbs (*Plantago lanceolata*) (Fajer at al. 1991), the growth rate of larvae of insect herbivores (*Junonia coensis*; Lepidoptera) fed with high CO_2 leaves was slower than ambient CO_2 leaves. It is also reviewed that defense level of most of plants may generally be enhanced by low soil fertility (Bryant et al. 1983, Price 1992, Lincoln et al. 1993, Orcutt and Nilsen 2000). These predictions are also supported by the results of a simulation experiment of annual herbs with varying nitrogen deposition and high CO_2 (Throop et al. 2004). In general, longevity of insect larvae is shorter fed with leaves at infertile soil condition than at fertile condition, as found in our results (birch, oak and maple) in Figs. 1 and 2. However, independent of treatments on alder, longevity of Erisan with alder was longer than that with oak and maple. Moreover, there was almost no difference in the longevity of Erisan fed with alder of all treatment.

Despite the large difference in N application between +N and –N, no difference in LMA and small difference in total phenolics were found in alder seedlings grown in all treatments (Figs. 3 and 5). The longevity and days of survival (ML_{50}) of Erisan larvae fed with alder leaves at 36 $PaCO_2$-N was longer than in the other three species at all treatments. Moreover, the ML_{50} with alder was similar to about 20~25 days independent of different CO_2 and soil fertilities. Again, why was the difference so small in the longevity of Erisan fed with alder leaves at differing CO_2 and nutrient levels?

Under natural conditions, leaves of alder have high nitrogen concentration and are usually grazed by leaf beetles (*Agelastica* sp.) because of the activities of symbiotic *Frankia* sp. in the root (Koike 1990, Koike et al. 1997). Why did alder have weaker defenses in infertile soil, in contrast to the other three species? A possible reason is high N_2 fixing ability of alder in infertile soil, and in addition, to maintain this property at high CO_2 condition in infertile soil. As a result, the nitrogen concentration in alder leaves was high even though they were grown in infertile conditions. These observations suggest that increased levels of CO_2 will weaken defense in alder by maintaining relatively high N concentration through enhancing the activity of nodules formed by *Frankia* sp. (Koike et al. 1997, Tobita et al. 2005). This indirect effect of symbiotic micro-organisms may vary greatly via the interaction between insect and plants (Ohgushi 2003). Moreover, all Erisan individuals tested were dead before they became pupae, even though alder leaves have less condensed tannin but high levels of total phenolics. There seems to be some toxic substance in the phenolics of alder leaves for Erisan.

The longevity of Erisan fed with birch and oak leaves grown in infertile soil was shorter, independent of CO_2 levels, which implies strong defense in the leaves (Fig. 2). This tendency was also seen in the high LAM value that indicates leaf toughness. The high C/N ratio in birch may imply a high concentration of starch or sucrose in leaves as well as an abundance of carbon-based secondary compounds. Birch had a higher C/N value of about 60 in leaves at 72 $PaCO_2$-N, but no marked increase in the amount of defense chemicals, such as phenolics and tannin, in leaves (Figs. 5, 6). This is partly explained by the greater capacity for accumulation of starch in birch leaves at high CO_2, and the dilution effect of leaf nitrogen (Koike et al. 1996). Similar traits have been reported for the paper birch (*B. papyrifera*) (Lindroth 1996) and the European white birch (*B. pendula*) (Kuokkanen et al. 2001). From the present results, we predict that quantitative chemical defense of birch and oak will increase in the coming high CO_2 environment, especially under infertile soil conditions. In addition, nitrogen input conditions (increase in N deposition) may mediate the effect of elevated CO_2 on the quantitative chemical defense of birch and oak.

The lowest longevity of Erisan was observed in individuals fed with maple leaves, irrespective of treatment. This was also found in a screening test in Betulacease involving maple (*Acer mono*) (Mastuki 2003) and representative species in northern Japan (Koike 1988, 1990). Although the C/N ratio of maple was similar to that of oak, the extent of chemical defense was larger than in the other three species at 72 $PaCO_2$ (Figs. 4~6). The amount of phenolics and tannin in maple were greater in infertile soil, and then slightly enhanced by elevated CO_2 (Figs. 5, 6). Chemical defense by maple should therefore increase in the coming high atmospheric CO_2 concentrations and nitrogen deposition.

In general, production of defense chemicals of trees is species specific, such that most late successional tree species have long-lived leaves with stronger defense than the early successional species native to northern Japan (e.g. Koike 1988, Matsuki 2003). Moreover, the production of defense chemicals of deciduous broadleaved trees involves the synthesis of carbon-based chemical compounds, so that production capacity depends strongly on nutrient conditions and atmospheric CO_2 levels during plant growth (e.g. Fajer et al. 1991, Lincoln et al. 1993). In fact the chemical defense capacity, as measured by the amount of total phenolics and condensed tannin in leaves of deciduous broadleaf trees, increases when plants are grown in poor soil conditions (Bryan et al. 1983, Coley et al. 1985). Meta-analysis of the defense capacity of woody species supports this observation (Koricheva at al. 1998), even though the carbon - nutrient balance (CNB) hypothesis might have limited relevance (Koricheva 2002).

The relative capacity for general defense of the four chosen deciduous broad-leaved trees is predicted by this experiment. However, many specialist herbivores in natural forests graze specific species. In north central American forests, the defense capacity of four tree species (aspen, birch, red oak and sugar maple) have been examined, under elevated CO_2 in combination with supply of nitrate nitrogen (NO_3^--N), against larvae of five specialist insect herbivores (Forest tent caterpillar, moths; gypsy, Cecropia, Luna, Polyphemus) (Lindroth 1996). Except for red oak, the growth rate of all moth species and caterpillars tested decreased when they were fed with leaves of these four species at high CO_2. However, the growth rate of gypsy moth fed with red oak leaves at high CO_2 increased. Total growth of the insect herbivores fed with aspen was unaffected by levels of atmospheric CO_2 and NO_3^- in soil. Although the gypsy moth is classified as a generalist, it may have high tolerance to red oak. So, specialism needs to be borne in mind when making predictions. Moreover, activities of insects may also change in the future thermal environment with increasing atmospheric CO_2 (Imura 1999).

The recovery capacity of tree species after grazing is species specific; for example, oak species (*Quercus serrata* and *Q. crispula*) have high recovery capacity after simulated defoliation when they were grown in fertile condition (Mizumachi et al. 2004). In seedlings of *Quercus serrata*, defoliated seedlings produced leaves with high nitrogen but lower concentration in phenolics and tannin (Hikosaka et al. 2005). Moreover, alders (i.e. *Alnus incana* and *A. glutinosa*) increase in light-saturated photosynthetic rate after grazing by herbivory (*Agelastica alni*) (Oleksyn et al. 1998). In our experiments, we avoided insect herbivores in phytotron. Therefore, we should know more information on the induced defense or compensation growth for predicting future defense traits under more natural conditions (Roth et al. 1998).

However, the life cycle of herbivores may also be affected by the growth temperature, and be inhibited by the activities of natural enemies such as birds (e.g. Imura 1999, Walker and Jones 2001, Bale et al. 2002). This report studies only the changes in leaf traits mediated by elevated CO_2 and an increase in nutrients. Further studies would be needed to predict the activities of insect herbivores and their interaction with plants in changing environments.

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Explanation of Figures

Figure 1. Time courses of survival of larvae of wild silkworms fed with leaves grown under combinations of two levels of CO_2 and soil fertilities. The curves were resulted in an average of six Erisan larvae. In maple, trace of the experimental results was overlapped.

Figure 2. Survival days in $\log_{10}$-transformed showing median longevity (ML_{50}) of larvae treated with leaves reared at differing CO_2 levels and soil fertilities.
Vertical bars indicate standard error (*n=4*).

Figure 3. Leaf mass per unit area (LMA) of four tree seedlings raised under combinations of two levels of CO_2 and soil fertilities.
Vertical bars indicate standard error (*n=4*).

Figure 4. C/N ratio of four tree seedlings treated with two levels of CO_2 and nutrient.

Vertical bars show standard error (*n=4*).

Figure 5. Concentration of total phenolics in leaves of four tree seedlings at two levels of CO_2 and nutrient.

Vertical bars indicate standard error (*n=4*).

Figure 6. Concentration of condensed tannin in leaves of four tree seedlings at two levels of CO_2 and nutrient.

Vertical bars indicate standard error (*n=4*).

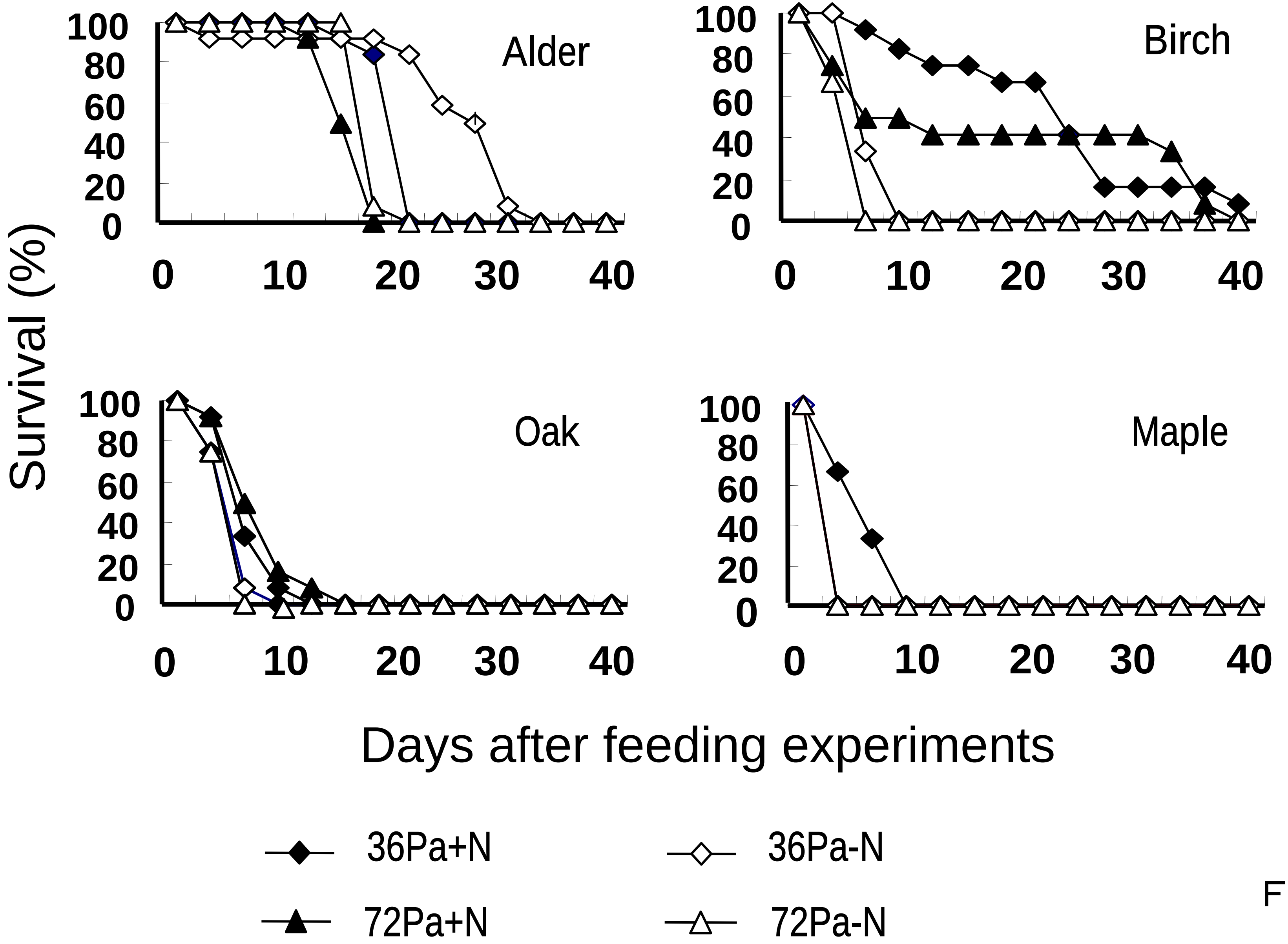
Survival (%)
Alder
Birch
Oak
Maple
100
80
60
40
20
0
0
10
20
30
40
Days after feeding experiments
36Pa+N
36Pa-N
72Pa+N
72Pa-N

Fig. 1

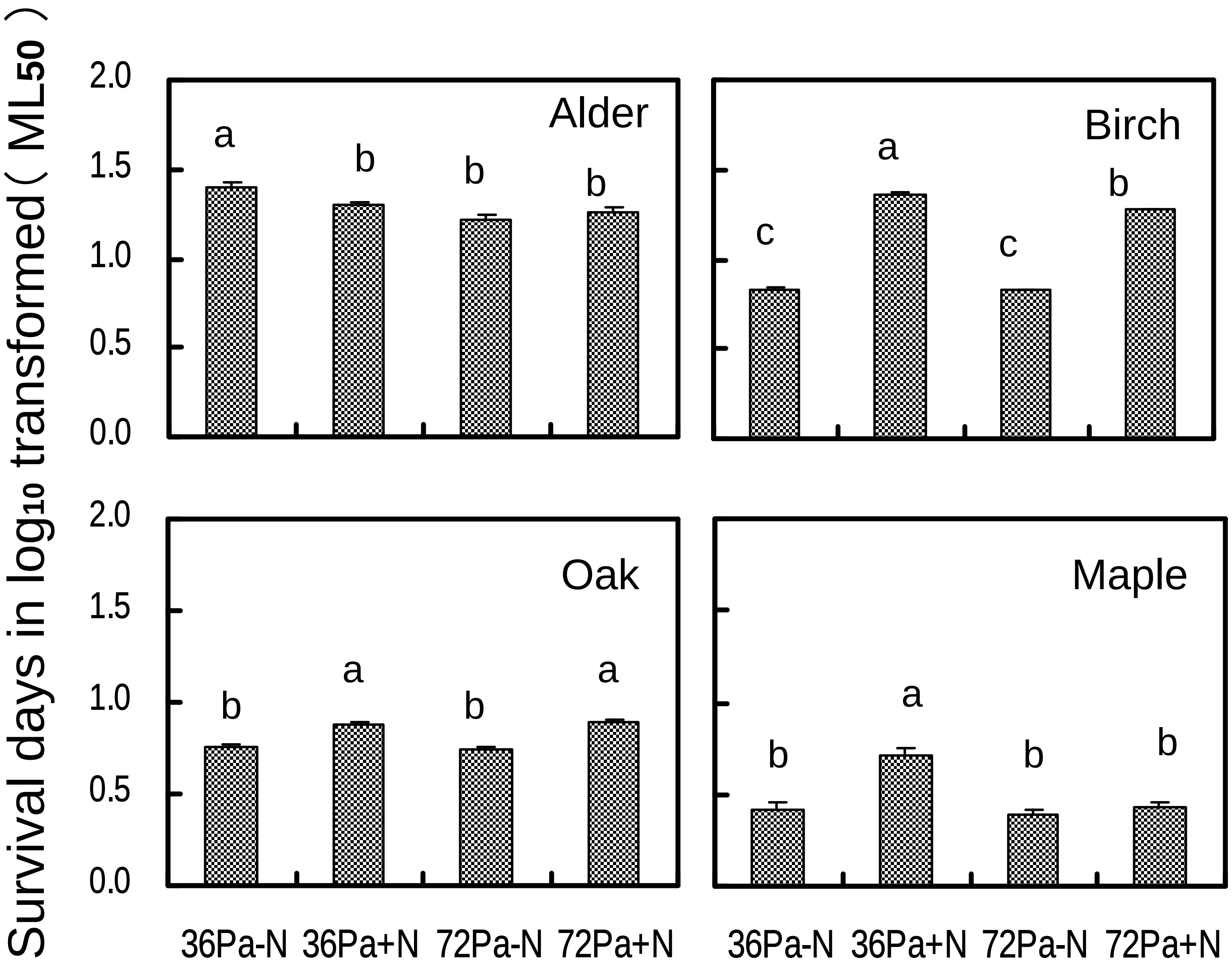
Survival days in log₁₀ transformed（ML50）
2.0
1.5
1.0
0.5
0.0
Alder
a
b
b
b
Birch
c
a
c
b
Oak
b
a
b
a
Maple
b
a
b
b
36Pa-N
36Pa+N
72Pa-N
72Pa+N
36Pa-N
36Pa+N
72Pa-N
72Pa+N

Fig. 2

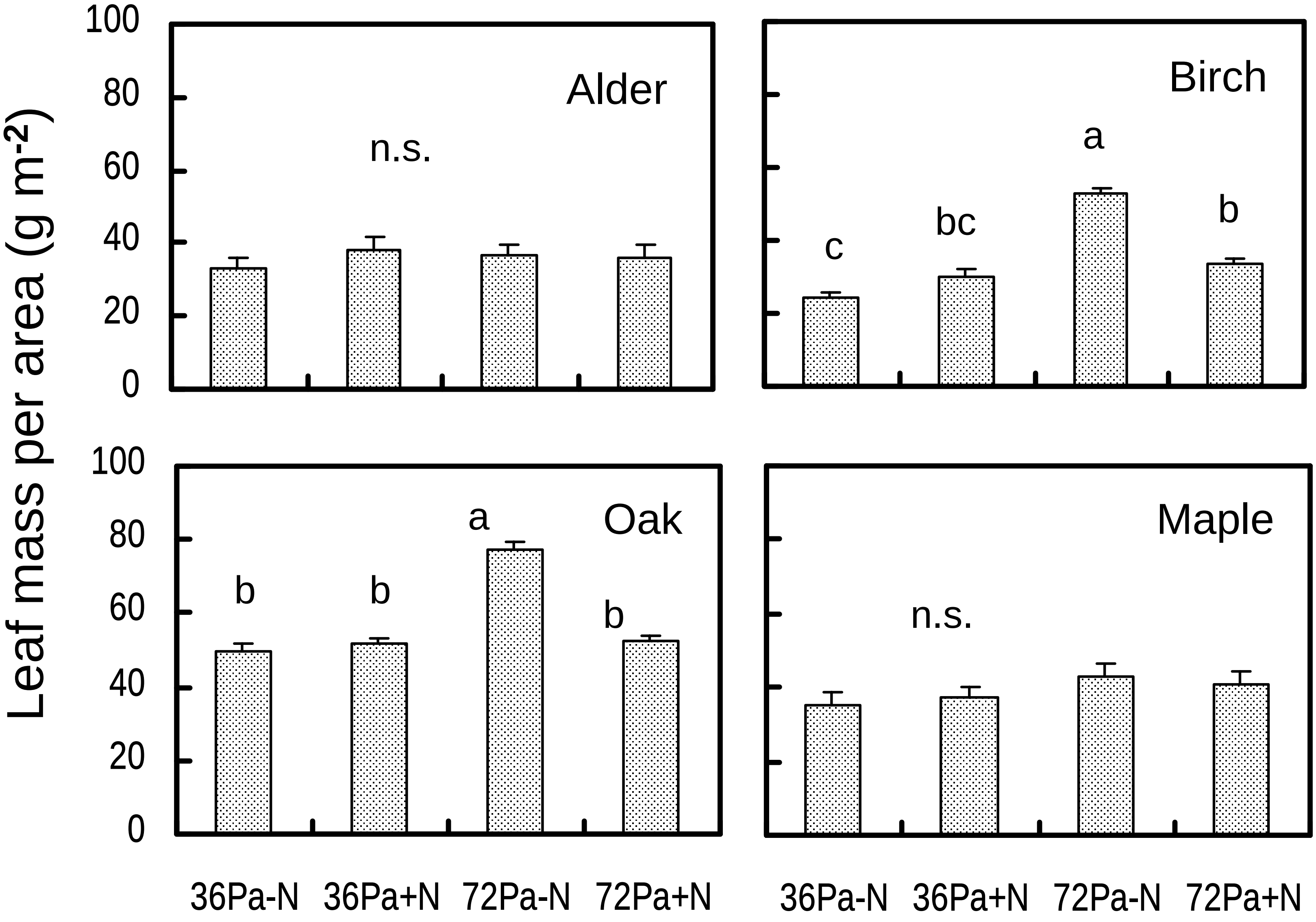Leaf mass per area (g m-2)
100
80
60
40
20
0
Alder
n.s.
Birch
c
bc
a
b
Oak
b
b
a
b
Maple
n.s.
36Pa-N
36Pa+N
72Pa-N
72Pa+N
36Pa-N
36Pa+N
72Pa-N
72Pa+N

Fig 3

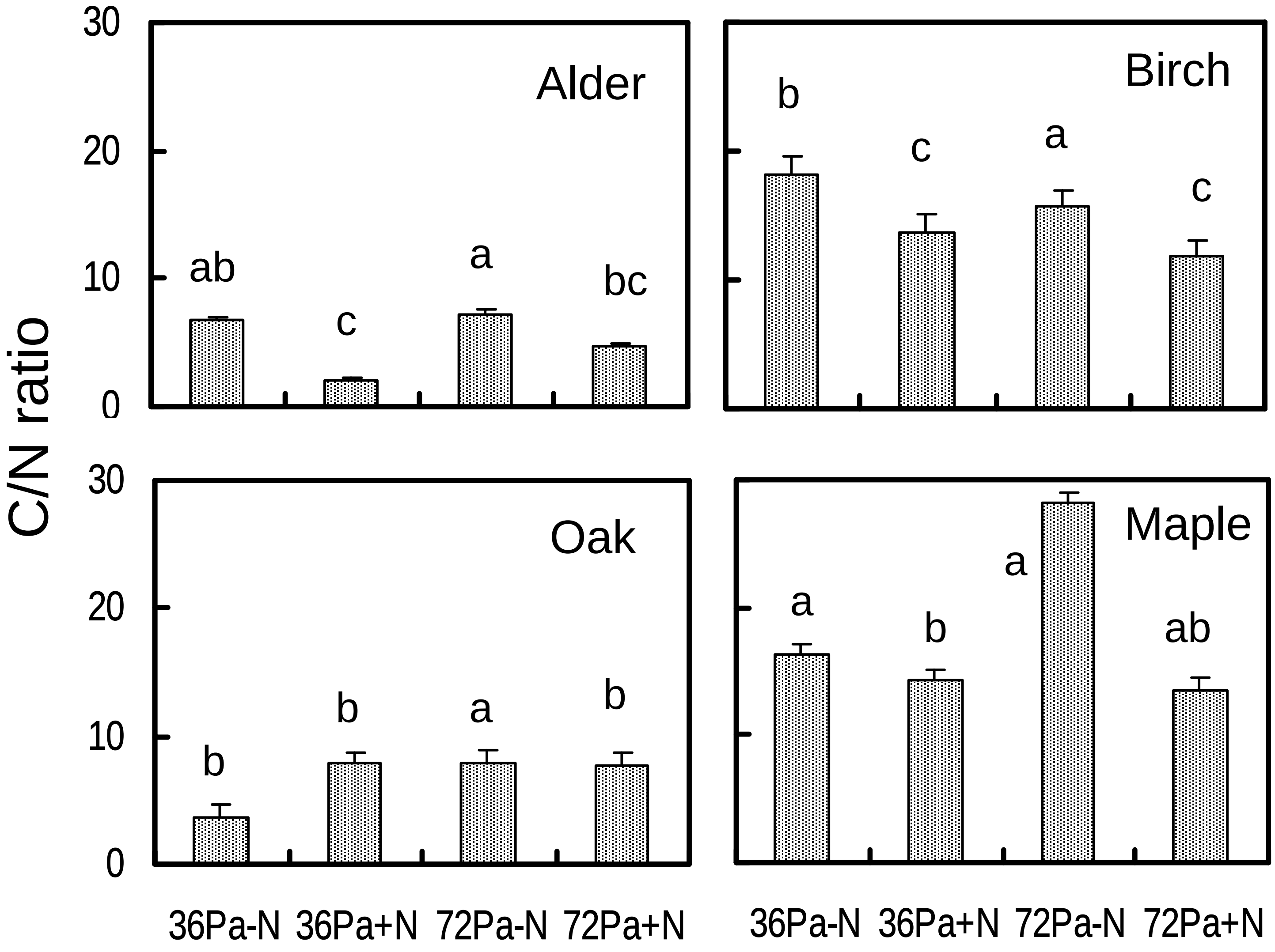
C/N ratio
Alder
Birch
Oak
Maple
30
20
10
0
ab
c
a
bc
b
c
a
c
b
b
a
b
a
b
a
ab
36Pa-N
36Pa+N
72Pa-N
72Pa+N

Fig 4

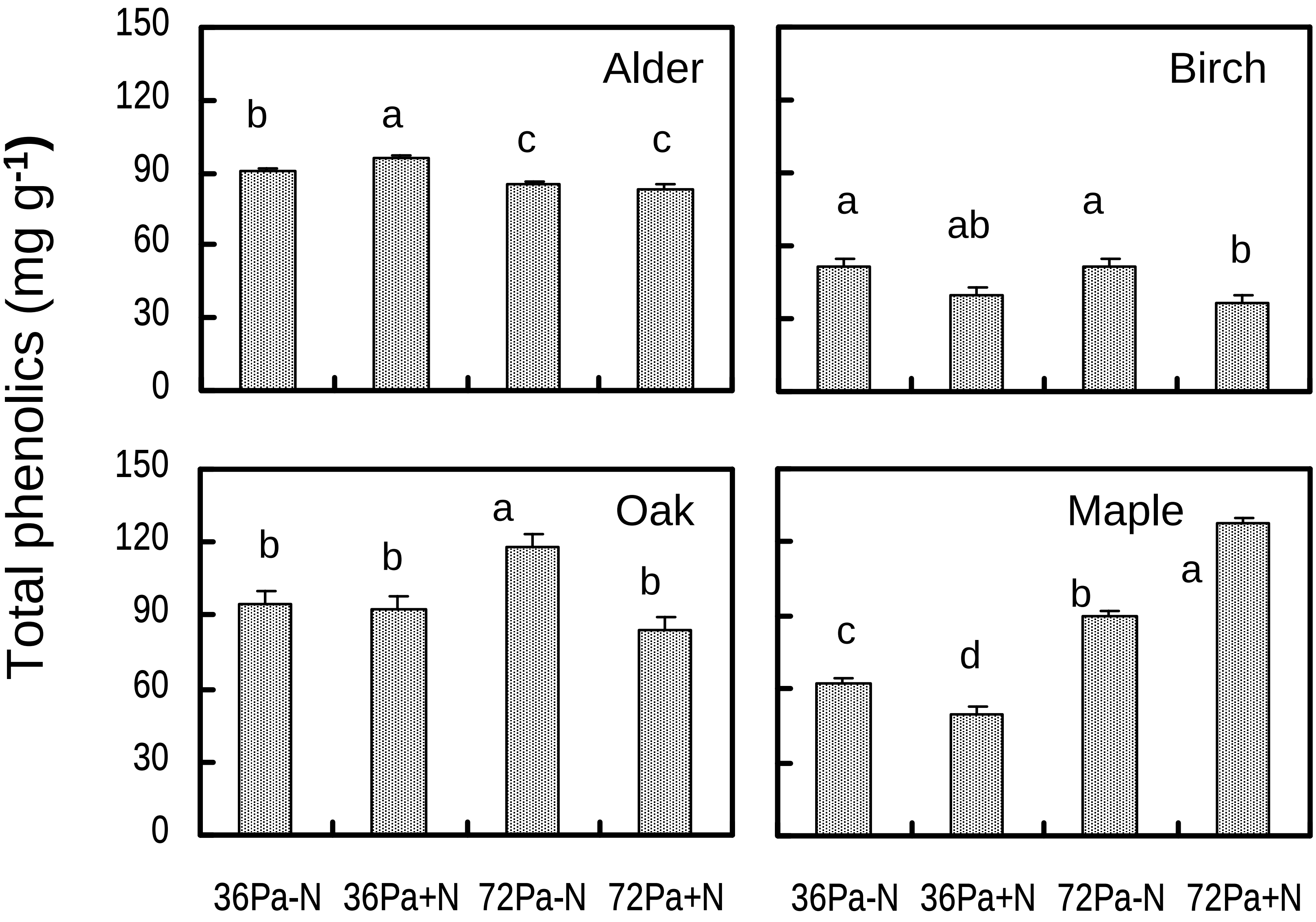
Total phenolics (mg g-1)
150
120
90
60
30
0
Alder
b
a
c
c
Birch
a
ab
a
b
Oak
b
b
a
b
Maple
c
d
b
a
36Pa-N
36Pa+N
72Pa-N
72Pa+N

Fig. 5

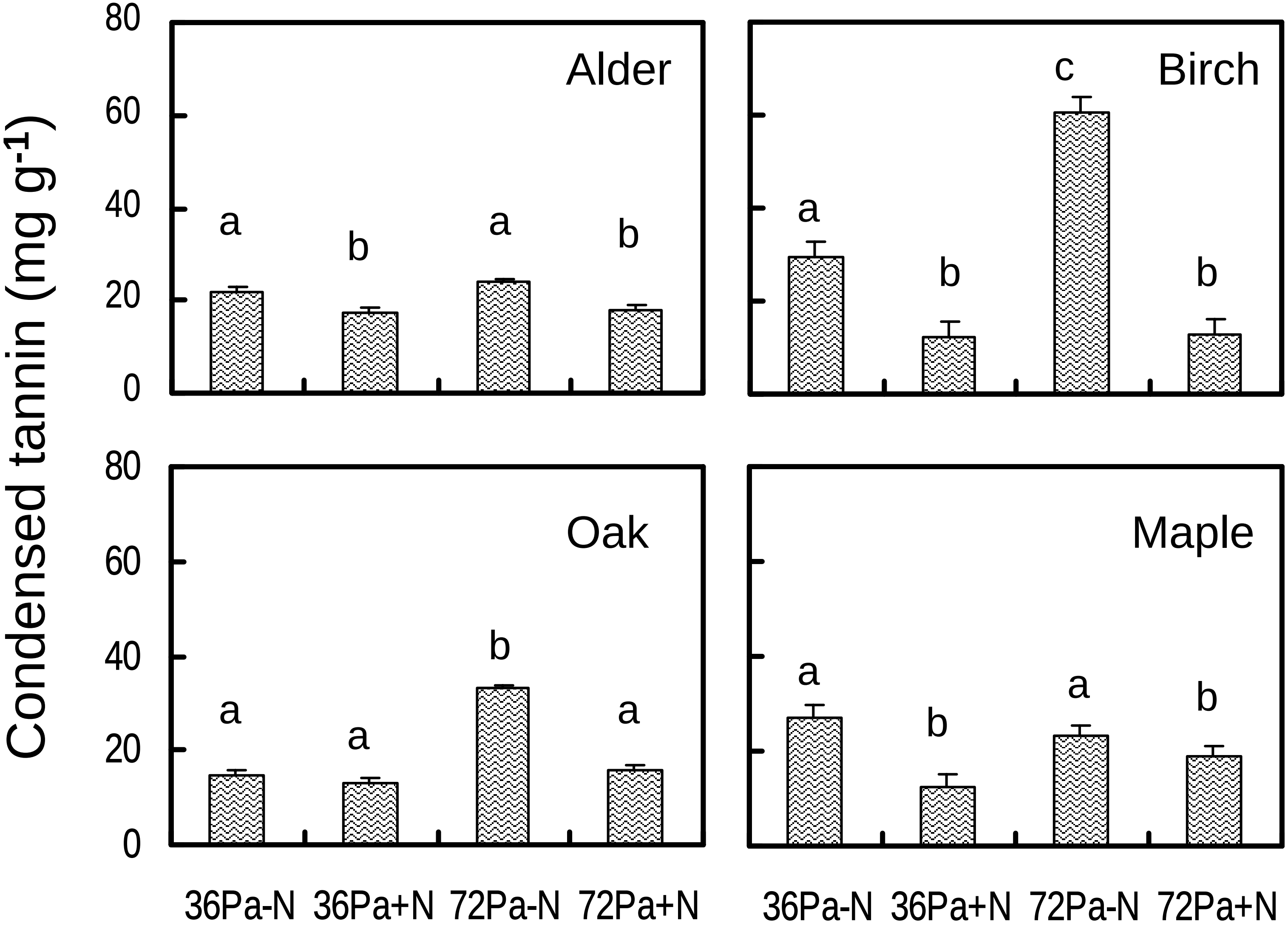
Condensed tannin (mg g^{-1})
80
60
40
20
0
Alder
a
b
a
b
Birch
a
b
c
b
Oak
a
a
b
a
Maple
a
b
a
b
36Pa-N
36Pa+N
72Pa-N
72Pa+N
36Pa-N
36Pa+N
72Pa-N
72Pa+N

Fig. 6