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**Original article**

# Consequences of an excess Al and a deficiency in Ca and Mg for stomatal functioning and net carbon assimilation of beech leaves

Michèle Ridolfi and Jean-Pierre Garrec*

Équipe Pollution Atmosphérique, Unité d'Écophysiologie Forestière, INRA Nancy, F-54280 Champenoux, France

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**Abstract** – Stomatal function and photosynthesis were investigated in beech seedlings submitted to excess Al, or/and to a deficiency in Ca and Mg. Excess Al in the nutrient solution promoted a decrease of Ca and Mg leaf contents, while K was increased. Stomatal responses to darkness, ABA and ambient $CO_2$ remained normal. In contrast, steady-state stomatal conductance in light was significantly smaller and correlated to a lower accumulation of K in the guard cells. Similar stomatal responses were observed for Ca-Mg deficient plants. In response to combined Al stress and low Ca and Mg nutrition, stomata remained almost insensitive to the different stimuli. The constancy in K guard cell concentration revealed a disturbance in K fluxes. Lower $CO_2$ assimilation rates and chlorophyll contents, on a leaf area basis, were recorded in response to all treatments. In conclusion, excess Al associated to low Ca and Mg nutrition lead to a strong stomatal dysfonction and reduced photosynthesis of beech seedlings.

**aluminium / mineral deficiencies / stomata / photosynthesis / *Fagus sylvatica***

**Résumé – Conséquences d'un excès d'Al et d'une carence en Ca et Mg sur le fonctionnement stomatique et l'assimilation nette de carbone de jeunes hêtres.** Cette étude présente les effets de l'aluminium, d'une double carence en Ca et Mg ou de la combinaison de ces deux traitements sur le fonctionnement stomatique et la photosynthèse de jeunes hêtres. Le stress aluminique a provoqué une carence en Ca et Mg, et une accumulation de K dans les feuilles. La réponse des stomates à l'obscurité, l'ABA et au $CO_2$ n'était pas perturbée. Par contre, les conductances stomatiques à la lumière étaient réduites et corrélées à une accumulation relative de K dans les cellules stomatiques plus faible. Les plants carencés en Ca et Mg présentaient des réponses stomatiques comparables à celles observées pour le traitement Al. Les plantes soumises à un stress aluminique et une carence calcico magnésienne présentaient une perte importante de sensibilité des stomates aux différents stimuli, associée à un dysfonctionnement des flux de K. Une réduction de la photosynthèse et des teneurs en chlorophylles, par unité de surface, fut enregistrée pour chaque traitement.
En conclusion, un excès d'aluminium associé à une nutrition minérale pauvre en Ca et Mg provoque un dysfonctionnement important des complexes stomatiques et une réduction de la photosynthèse.

**aluminium / carences minérales / stomate / photosynthèse / *Fagus sylvatica***

* Correspondence and reprints
Tel. 33-3 83 39 40 97; Fax. 33-3 83 39 40 69; e-mail: garrec@nancy.inra.fr

**Abbreviation**

ABA, abscisic acid;

Chl, chlorophyll;

A, net $CO_2$ assimilation rate ($\mu mol\ m^{-2}\ s^{-1}$);

$g_w$, stomatal conductance to water vapour ($mmol\ m^{-2}\ s^{-1}$);

$c_a$, $c_i$, $CO_2$ mole fractions in the air and in the sub-stomatal spaces ($\mu mol\ mol^{-1}$);

PPFD, photosynthetic photon flux density ($\mu mol\ m^{-2}\ s^{-1}$);

SD, standard deviation.

## 1. INTRODUCTION

The role of nutrient imbalance in the worsening of tree health has been established in the Ardennes forests [47]. These ecosystems are characterized by acid brown soil with a low base cation status [23]. Furtermore, they may be subjected to acidifying substances and as a consequence to increased free aluminium in the soil solution. Excess $Al^{3+}$ is well known to affect tree vitality. The initial symptom of Al toxicity is the inhibition of root elongation, which has been proposed to be caused by a number of different mechanisms, including Al interactions within the cell wall, the plasma membrane or the symplast [for a review see 20]. At shoot level, leaf necrosis as a visible symptom of Al stress, was found to be accompanied by decreasing chlorophyll concentrations and photosynthetic rates in *Picea abies* [32]. Moreover, Al has generally been found to decrease transpiration rates. This was attributed to reduced absorbing surfaces [37], root-water permeability [48], or stomatal aperture [15, 33]. In contrast, Schlegel and Godbold [32] observed enhanced transpiration rates of spruce needles due to Al. The impact of Al on plant water balance appears to be complex, and therefore requires further investigations.

Although the mechanism of Al toxicity has not yet been completely established, it may be the result of both primary and secondary effects of Al. Several investigations have shown that many tree species respond to Al exposure with changed mineral uptake [2, 6, 11, 41, 42, 43]. An Al-induced reduction in Ca, Mg and P concentrations was reported in roots and shoots of European beech [5, 6, 39]. In contrast, K amounts in leaf tissues were found to increase with increasing Al concentration in the rhizosphere [3, 6]. It is well established that mineral ions play a key role in stomatal function, which control both leaf transpiration and carbon assimilation. While potassium is the main cation involved in the osmotic build-up required for stomatal opening, cytosolic free calcium serves in the signal transduction pathway linking the variations of environmental conditions to stomatal movements [19, 26, 28 and 40]. Schnabl and Ziegler [33] found that 1 mM $Al^{3+}$ inhibits stomatal opening in illuminated epidermal strips of *Vicia faba*, by preventing $K^+$ accumulation and starch mobilization in the guard cells. Ridolfi et al. [30] reported a lack of stomatal response to darkness, and a reduced ABA-induced stomatal closure in Ca-deficient plants of *Vicia faba*. In a tree specie (*Quercus robur*), a calcium deficieny did not affect the stomatal reactivity to darkness and ABA supply; but the light stomatal opening was significantly reduced and accompanied by a lower net carbon assimilation [31].

Based upon these considerations, our objective was to i) analyse the effects of Al on stomatal function and photosynthesis of the European beech, and ii) to estimate the role of Al-induced nutrient imbalance in potential stomatal disorders. Therefore, beech seedlings were submitted to excess Al, to reduced Ca and Mg nutrition, or to combined treatments. The concentrations of Al, Ca, Mg and K in the leaf cells were measured by X-ray microanalysis. We assessed potential disorders in stomatal reactivity to different stimuli: i.e. darkness, light, exogenous ABA and $CO_2$ mole fraction in the air. We also checked K concentrations in the guard cells of closed and open stomata. Photosynthesis was estimated by determining chlorophyll concentrations in the leaves and net $CO_2$ assimilation rates.

## 2. MATERIALS AND METHODS

### 2.1. Plant growth

Beech seedlings were bred at the Center of Forest Research, Section Ecopedology, Faculty of Agronomy (Gembloux, Belgium).

Beech-nuts (origin: Bertrix Forest, Ardennes, Belgium) stored at –20 °C and at 9% relative humidity [45], were germinated in the laboratory during March 1992. After germination, seedlings were grown outside under a glass roofed shelter, in semi-hydroponic culture systems. Pots were filled with calibrated alluvial, acid washed coarse sand (0.4 – 0.8 mm). They were equipped with a device allowing drainage and control of the water level. Each pot contained 6 plants and was irrigated two to three times a week. Three times during plant growth, the substrate was washed with distilled water before adding the nutrient solution. Plants were kept under optimal conditions until end of May, and then subjected to Al stress, to a deficiency in Ca and Mg or to combined treatments.

The solution for control plants was as follows (pH, 4.5): $H_3BO_3$, 0.461 µM; $MnCl_2$, 0.015 µM; $ZnSO_4$ ($7H_2O$), 0.767 µM; $MoO_3$, 0.208 µM; $CuSO_4$ $5H_2O$, 0.321 µM; EDTA $Fe_{III}$ Na, 0.11 mM; $KH_2PO_4$, 0.1 mM; $K_2SO_4$, 0.1 mM; $CaCl_2$ $2H_2O$, 0.6 mM; $MgSO_4$ ($7H_2O$), 0.2 mM; $(NH_4)_2$ $SO_4$, 0.75 mM. Ca and Mg deficiencies were induced by decreasing $CaCl_2$ ($2H_2O$) to 9.97 $10^{-2}$ mM, and $MgSO_4$ ($7H_2O$) to 2.51 $10^{-2}$ mM (pH, 4.5). Aluminium was supplied at a concentration of 0.37 mM ($Al_2$ $(SO_4)_3$ $18H_2O$, 0.183 mM), and the pH was adjusted to 3.8 with HCL 0.1 N.

During July, the plants were transferred to INRA-Nancy (Champenoux, France). All experiments were conducted during two weeks in a climate chamber with the following day/night conditions: 14/10 h; RH, 55%; air temperature, 22/20 °C; PPFD at the top of the plants around 300 µmol $m^{-2}$ $s^{-1}$.

### 2.2. Stomatal movements and photosynthesis

Stomatal density was measured on the abaxial side of six leaves (from six different plants) per treatment using a scanning electron microprobe (Cambridge Instruments, Cambridge, UK). For each leaf, stomata were counted on six squares of 0.04 $mm^2$.

Stomatal movements were followed from changes in stomatal conductance. Stomatal conductance to water vapor ($g_w$) was monitored by means of a diffusive porometer (Delta-T-Devices, Cambridge, UK) under darkness (measured at predawn), after 4h of light supply (PPFD around 300 µmol $m^{-2}$ $s^{-1}$) or after exogenous ABA supply. ABA (±-2-cis, 4-trans-abscisic acid, Aldrich-Chemie, Steinheim, Germany) was taken up by the plant xylem. The stems of four plants per treatment were cut under water, and after 1h of irradiance (PPFD around 300 µmol $m^{-2}$ $s^{-1}$), the shoots were transferred to a tube containing an aqueous solution of ABA ($10^{-3}$ M). The relationships between gw and ambient $CO_2$ (c.a.) were established on four plants per treatment by means of a portable photosynthesis chamber (LI 6200, LI-COR Inc., Lincoln, Nebraska) as described by McDermitt et al. [29]. Four to five leaves per plant were enclosed into a 4 l assimilation chamber, and the $CO_2$ mole fraction ($c_a$) was increased to about 950 µmol $mol^{-1}$ by breathing into the chamber. $g_w$ was measured when decreasing $c_a$ from 900 to 50 µmol $mol^{-1}$. $CO_2$ mole fraction in the chamber was lowering with a soda lime scrub.

Net $CO_2$ assimilation rates (A) were recorded at $c_a$ of 350 µmol $mol^{-1}$ and PPFD of 250 µmol $m^{-2}$ $s^{-1}$. Both A and the sub-stomatal $CO_2$ concentration ($c_i$) were calculated following the equations of Von Caemmerer and Farquhar [44]. Chlorophylls were extracted from eight leaf disks (3 $cm^2$, from eight different plants) per treatment in 5 $cm^3$ of dimethyl-sulphoxide (DMSO) for 90 min at 65 °C and determined spectrophotometrically [4].

### 2.3. Mineral X-ray microanalysis

Parallel to stomatal conductance measurements, under both light and darkness, leaves were sampled for mineral X-ray microanalysis. To prevent any exchange of diffusive ions (i.e. $K^+$ and $Cl^-$), the leaves were immediately frozen in liquid nitrogen. Leaf sections of 2 mm width were cut off at –30 °C by means of a razor blade. Samples were then freeze-dried at –10 °C, as previously described [13], and carbon coated (metallizer Balzer's CED/020, Boiziau distribution, Selles sur Cher, France). Cell concentrations of Al, K, Ca and Mg were measured with a Stereoscan 90 electron microprobe fitted with an AN 10000 10/25 energy-dispersive-analyser (Cambridge Instruments, Cambridge, UK) in eight leaves (from eight different plants) per treatment. For each leaf, three cells were analysed in the different leaf tissues. Analysis was performed in the scanning mode with a 15 KV acceleration voltage and a tilt angle of 45° for 100 s in the middle of the cells. Spectra were treated with the program ZAF4 - FLS (Cambridge Instruments, Cambridge, UK) and the results were expressed in mg $g^{-1}$ DW leaf tissue.

Potassium is mainly located in the cell vacuole. Therefore, X-ray microanalysis at a cell level allow a good estimation of $K^+$ fluxes between the guard cells and the epidermal cells. On the other hand, such investigation gives no information about $Ca^{2+}$ and $Al^{3+}$ concentrations in the apoplast or in the cytosol.

### 2.4. Statistical treatment

The effects of nutrition treatments were investigated by analysing the variance on the base of the Fisher test. Least significant differences (Student PLSD, $p < 0.05$) were then calculated to range means values. Data were also examined for significant interactions ($p < 0.05$) between excess Al and a deficiency in Ca and Mg.

## 3. RESULTS

### 3.1. Element concentrations of leaf cells

The distribution of Al in the different leaf cells is presented in *figure 1*. In control plants, a concentration of 0.94 mg $gDW^{-1}$ Al was recorded in the guard cells. In abaxial epidermal cells, Al concentration was only at

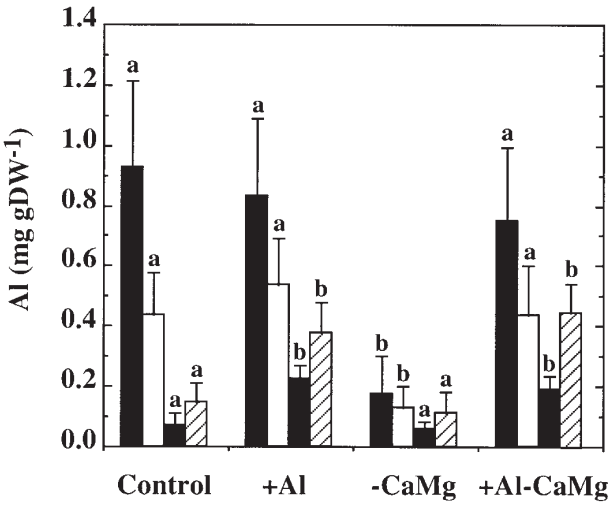


**Figure 1.** Aluminum concentrations of the different leaf cells: black, guard cells; white, abaxial epidermal cells; grey, spongy parenchyma cells, stripe, palisade parenchyma cells. (mean ± SD; $n$ = 8 leaves from 8 different plants; values with different letters are significantly different at $p < 0.05$).

50% of the guard cell value. The lowest concentrations of Al were observed in the parenchyma cells, the palisade parenchyma always showing higher Al concentrations than the spongy parenchyma. Excess Al in the nutrient solution (+Al and +Al-CaMg plants) did not increase significantly Al concentrations in guard cells and epidermal cells. In contrast, a significant increase in Al content, up to about twice the control value, was recorded in both parenchyma types. Regarding -CaMg plants, a similar trend was observed in the distribution of Al in the leaf cells. Nevertheless, Al concentrations in guard cells and epidermal cells represented about 19% and 29% of the control values, respectively. In parenchyma cells, Al concentrations were similar to those of control plants. No interaction between excess Al and Ca-Mg deficiency was recorded.

*Table I* presents K, Ca and Mg concentrations in the abaxial epiderm and in both parenchyma. Ca-Mg depletion in the nutrient solution resulted in lower Ca and Mg in all cells, while K was not affected. Excess Al (+Al and +Al-CaMg plants) induced a decrease of Ca and Mg in all leaf tissus, which was comparable to the one recorded with the -CaMg treatment. In contrast, K concentrations were significantly increased by Al stress in all leaf cells. No interaction between excess Al and a deficiency in Ca and Mg was observed on K, Ca and Mg concentrations for the different leaf cells.

### 3.2. Stomatal response and K fluxes under darkness or irradiance *(figure 2)*

Beech seedlings from the different treatments displayed similar leaf stomatal densities *(table II)*. Therefore, differences in leaf conductance ($g_w$) resulted from differences in stomatal aperture.

In control plants, mean stomatal conductance of dark-adapted leaves was around 30 mmol $m^{-2}$ $s^{-1}$. Four hours

**Table I.** Potassium, calcium and magnesium concentrations in the different leaf cells. (mean ± SD; $n$ = 8 leaves from 8 different plants; value with different letters are significantly different at $p < 0.05$).

| | Element concentrations (mg $gDW^{-1}$) | | |
|---|---|---|---|
| | **K** | **Ca** | **Mg** |
| | | ***Abaxial epidermal cells*** | |
| **Control** | 9.3 ± 2.2 [a] | 11.3 ± 2.2 [a] | 4.0 ± 0.9 [a] |
| **+ Al** | 17.4 ± 2.4 [b] | 7.5 ± 1.8 [b] | 1.6 ± 0.6 [b] |
| **– CaMg** | 9.1 ± 3.4 [a] | 7.8 ± 1.2 [b] | 1.5 ± 1.1 [b] |
| **+Al-CaMg** | 15.6 ± 2.2 [b] | 8.0 ± 1.3 [b] | 1.0 ± 1.0 [b] |
| | | ***Spongy parenchyma cells*** | |
| **Control** | 6.2 ± 1.1 [a] | 5.3 ± 0.4 [a] | 1.5 ± 0.3 [a] |
| **+ Al** | 12.4 ± 2.1 [b] | 2.7 ± 0.6 [b] | 0.7 ± 0.3 [b] |
| **– CaMg** | 5.2 ± 1.4 [a] | 3.0 ± 0.3 [b] | 0.4 ± 0.3 [b] |
| **+Al-CaMg** | 10.9 ± 2.4 [b] | 2.5 ± 0.7 [b] | 0.6 ± 0.4 [b] |
| | | ***Palisade parenchyma cells*** | |
| **Control** | 7.0 ± 1.1 [a] | 9.2 ± 1.8 [a] | 2.5 ± 0.5 [a] |
| **+ Al** | 10.1 ± 1.3 [b] | 6.1 ± 1.3 [b] | 0.8 ± 0.2 [b] |
| **– CaMg** | 6.4 ± 2.5 [a] | 5.5 ± 1.8 [b] | 0.7 ± 0.5 [b] |
| **+Al-CaMg** | 11.9 ± 2.4 [b] | 5.9 ± 1.6 [b] | 0.8 ± 0.4 [b] |

**Table II.** Chlorophylls concentrations, stomatal densities, net $CO_2$ assimilation rates (A; PPFD = 250 µmol $m^{-2}$ $s^{-1}$) and $CO_2$ mole fractions in the sub stomatal spaces ($c_i$). (mean ± SD; value with different letters are significantly different at $p < 0.05$).

| | **Chl a** | **Chl b** (mg $dm^{-2}$, $n$ = 8 leaves) | **Chl a / Chl b** |
|---|---|---|---|
| **Control** | 2.25 ± 0.24 [a] | 0.49 ± 0.11 [a] | 4.72 ± 0.98 |
| **+ Al** | 1.48 ± 0.61 [b] | 0.31 ± 0.11 [b] | 4.74 ± 0.59 |
| **– CaMg** | 1.12 ± 0.23 [b] | 0.25 ± 0.06 [b] | 4.50 ± 0.81 |
| **+ Al – CaMg** | 1.29 ± 0.20 [b] | 0.28 ± 0.07 [b] | 4.67 ± 0.64 |
| | **Stomata mm$^{-2}$** $n$ = 6 leaves | **A** (µmol $m^{-2}$ $s^{-1}$) | **ci** (µmol $mol^{-1}$) $n$ = 4 plants |
| **Control** | 257 ± 43 | 2.57 ± 0.32 [a] | 329 ± 4 |
| **+ Al** | 245 ± 45 | 1.53 ± 0.20 [b] | 334 ± 5 |
| **– CaMg** | 271 ± 52 | 1.45 ± 0.40 [b] | 333 ± 4 |
| **+ Al – CaMg** | 261 ± 63 | 0.60 ± 0.28 [c] | 340 ± 6 |

irradiance increased $g_w$ up to 150 mmol $m^{-2}$ $s^{-1}$ and K concentration of the guard cells up to 17.9 *vs.* 8.8 mg $gDW^{-1}$ in darkness. As a result, the ratio guard cells/epidermal cells for K contents (Kgd/Kep) was enhanced from 0.9 to 1.9.

Ca and Mg low nutrition did not affect the stomatal response to darkness: $g_w$, guard cell K concentration and Kgd/Kep were similar to the control values. On the other hand, steady-state $g_w$ in light was significantly lower (107 mmol $m^{-2}$ $s^{-1}$) and correlated to lower Kgd/Kep (1.4), as a result of smaller K accumulation in the guard cells.

Excess Al resulted in an increase in K concentrations in the guard cells, as previously observed in the epidermal cells (*table I*). Therefore, Kgd/Kep remained closed to controls, and was even lower for the light adapted state. This smaller relative accumulation of potassium was associated to lower $g_w$ for light condition (116 mmol $m^{-2}$ $s^{-1}$). It is notworthy that, despite the difference in absolute K contents, Kgd/Kep and $g_w$ were similar for +Al and –CaMg plants.

The seedlings submitted to combined Al stress and a deficiency in Ca and Mg were characterized by high $g_w$ in darkness: 80 *vs.* 30 mmol $m^{-2}$ $s^{-1}$ in control leaves. Light supply promoted only a slight increase in $g_w$ up to 97 mmol $m^{-2}$ $s^{-1}$. X-ray microanalysis showed a lack of K accumulation between dark and light conditions. Kgd/Kep remained constant and similar to the value recorded in control dark-adapted leaves, i.e. 0.9.

### 3.3. Stomatal response to ABA

Stomatal responses to an application of exogenous ABA *via* the transpiration stream are presented in *figure 3*. Control leaves showed a decrease in stomatal conductance 25 min after ABA supply, and $g_w$ stabilized to 38% of the initial value after 100 min +Al and –CaMg treatments affected neither the time course of stomatal response to ABA, nor the magnitude of stomatal closure. In contrast, +Al–CaMg plants exhibited a limited ABA-induced stomatal closure not lower than 67% of the initial value.

### 3.4. Stomatal response to $CO_2$

Stomatal responses to changing $CO_2$ mole fraction in the air ($c_a$) are presented in *figure 4*. Control plants showed increased $g_w$ of 29% when $c_a$ was decreased from 900 to 50 µmol $mol^{-1}$. In +Al, –CaMg and +Al–CaMg plants, $g_w$ at $c_a$ = 900 µmol $mol^{-1}$ was significantly lower than in controls (–36%). Stomata of both +Al and –CaMg plants remained wide open with lowering $c_a$. On the other hand, combined treatments hardly reduced the stomatal response to $CO_2$. The increase in $g_w$ at $c_a$ 50 µmol $mol^{-1}$ represented only 16% of the value recorded at 900 µmol $mol^{-1}$ for +Al–CaMg plants.

### 3.5. Net $CO_2$ assimilation

Chlorophyll concentrations on a leaf area basis are presented in *table II*. A significant and similar reduction

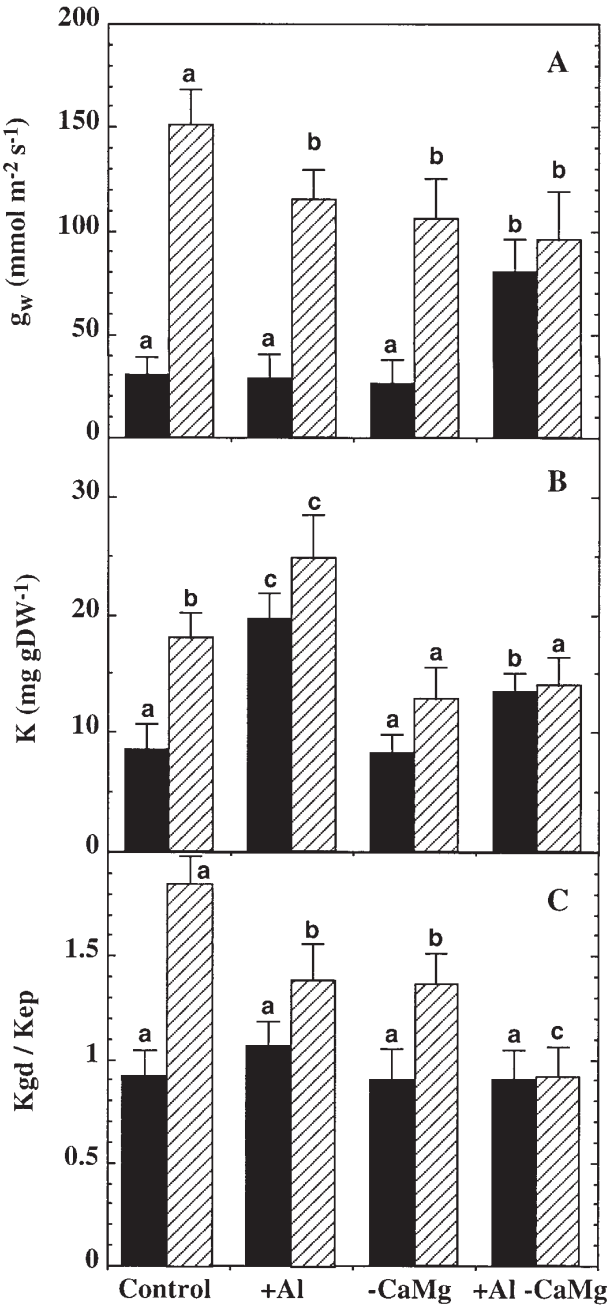


**Figure 2.** A) Steady state stomatal conductances to water vapour ($g_w$), B) potassium concentration in the guard cells and C) ratio between guard cells and epidermal cells concentrations in K; under darkness (black) and after 4 hours of light supply (stripe). (PPFD = 300 μmol m$^{-2}$ s$^{-1}$; mean ± SD; $n$ = 8 leaves from 8 different plants; values with different letters are significantly different at $p < 0.05$).

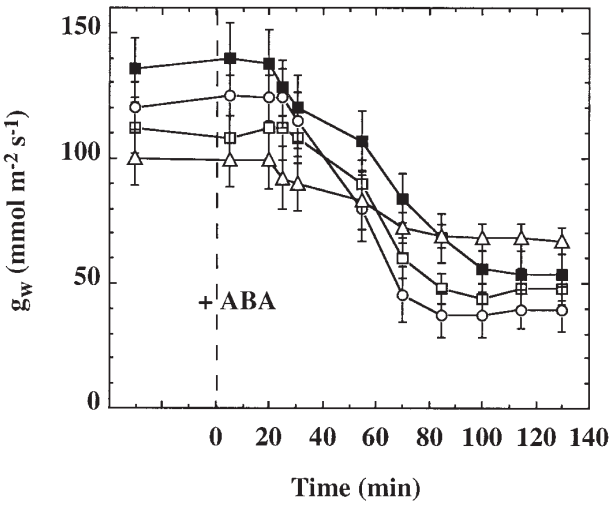


**Figure 3.** Change in stomatal conductances to water vapor ($g_w$) in response to exogenously applied ABA ($10^{-3}$ M): black squares, control; white disks, +Al; white squares, –CaMg; white triangle, +Al –CaMg. All walues are presented as mean ± SD; $n$ = 4 leaves from 4 different plants; (PPFD = 300 μmol m$^{-2}$ s$^{-1}$).

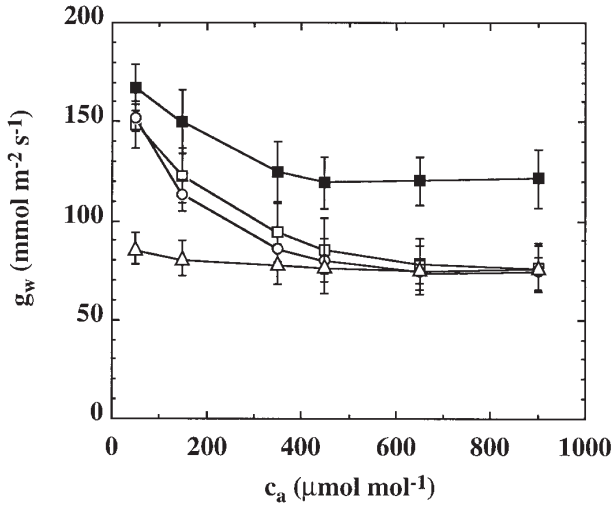


**Figure 4.** Change in stomatal conductances to water vapor ($g_w$) with decreasing $CO_2$ mole fraction in the air ($c_a$): black squares, control; white disks, +Al; white squares, –CaMg; white triangle, +Al –CaMg. All walues are presented as mean ± SD; $n$ = 4 plants (PPFD = 250 μmol m$^{-2}$ s$^{-1}$).

of both chl a and chl b concentrations was recorded in the leaves of +Al, –CaMg and +Al–CaMg plants to about 40% of the control values. The ratio chl a/chl b was never affected. Mean net $CO_2$ assimilation rates (A), on a leaf area basis, were significantly depressed in all treated plants. The reduction in A was not significantly different between +Al (–31%) and –CaMg (–43%) treatments. An interaction between excess Al and a deficiency in Ca and Mg was calculated for +Al–CaMg plants (–70%). The decrease in A was accompanied by a constancy of the calculated sub-stomatal $CO_2$ mole fraction ($c_i$). On a chlorophyll a concentration basis, A for +Al (1.2 μmol $gChl^{-1}$ $s^{-1}$) or –CaMg (1.0 μmol $gChl^{-1}$ $s^{-1}$) leaves were not different from control (1.1 μmol $gChl^{-1}$ $s^{-1}$). In +Al –CaMg plants, A was reduced to one half of the control: 0.5 μmol $gChl^{-1}$ $s^{-1}$.

## 4. DISCUSSION

Stomata allow water loss by transpiration and the entry of $CO_2$ into the leaf for photosynthetic carbon fixation. Fine control of stomatal conductance is vital so that tree neither dessicates nor becomes starved for $CO_2$. In control beech seedlings, light as expected triggered stomatal opening while darkness, exogenous ABA and high $CO_2$ concentration in the air reduced the stomatal conductance. X-ray microanalysis showed the occurrence of K fluxes with stomatal movements in beech. The transition from darkness to light promoted an increase in stomatal conductance accompanied by a build-up in potassium guard cell concentration (measured after 4h of irradiance). Such accumulation of K in the guard cells upon illumination has been well documented in herbaceous plants [22, 24, 25]. The aim of this work was to assume whether free aluminium in the rhizosphere may affect beech vitality *via* a disturbance in stomatal regulation and leaf carbon assimilation.

In beech seedlings exposed to aluminium, Al accumulated in the parenchyma, and palisade cells always showed higher Al concentration than in the spongy cells. The highest concentrations were always recorded in the guard cells, and may result from an accumulation of Al *via* the transpiration stream. +Al and +Al–CaMg plants showed similar Al concentration. It should be remember that X-ray microanalysis were performed on dehydrated leaf sections and at cell level. Therefore, it is impossible to distiguish any difference in Al cell localisation nor Al speciation between the two treatments. Al promoted a reduction of Ca and Mg levels in all leaf tissues, which was comparable to those recorded with decreasing Ca and Mg nutrition. With all treatments, cell concentrations of Mg were below the deficiency threshold for this element (i.e. 1 mg $gDW^{-1}$, [8]). The spongy parenchyma cells also showed a severe deficiency in calcium, and the cells of the palissade parenchyma were decreased closed to the deficency level estimated at leaf level (5 mg $gDW^{-1}$, [8]). We assumed that the seedlings were also deficient in calcium. Combined stresses (+Al–CaMg plants) did not result in a further reduction in Ca and Mg leaf amounts. On the other hand, potassium concentration was significantly increased by Al stress in all leaf cells. Similar Al effects on the mineral balance has been described by several authors for *Fagus sylvatica* [3, 6], *Quercus rubra* [10, 21] and *Picea abies* [16, 32]. This study confirms that Al reduces the uptake and the translocation of Ca and Mg. The raise in K leaf concentration could not be attributed to the depletion in Ca and Mg. Indeed, for –CaMg plants, K concentrations remained similar to the control values.

With regards to stomatal regulation, the main questions were as follows: i) Does Al accumulation in leaf tissues inhibit the light-induced $K^+$ influx into the guard cell vacuole? ii) What is the consequence of Al-induced K accumulation in the leaf cells on stomatal aperture? and iii) What is the consequence of Al-induced Ca deficiency on the signal transduction pathway leading to stomatal closure?

With calcium deficiency, Ridolfi et al. [30] observed a reduced stomatal sensitivity to both darkness and ABA in *Vicia faba.* The authors hypothesized that reduced calcium availability at leaf level probably affects the increase in cytosolic [$Ca^{2+}$] required for stomatal closure. Indeed, ABA [9, 27] and darkness [36] are known to induce stomatal closure mainly *via* a transient increase of cytosolic-free $Ca^{2+}$ in the guard cells, which in turn inhibits proton efflux [18] and $K^+$ uptake [7], and activates anion efflux [34]. For beech seedlings, Ca and Mg depletion did not affect stomatal response to the different closing stimuli: darkness, ABA supply and high $CO_2$ concentration in the air. Similar results were obtained on Ca deficient oaks [31]. Alternative explanations could be i) sufficient amount of free calcium in the vicinity of the guard cells and ii) the existence of a Ca independent signal transduction pathway for these tree species. The occurrence of several transduction routes leading to stomatal closure has been previously speculated in *Commelina communis* [1, 14] and *Vicia faba* [30].

On the other hand, steady state stomatal conductances ($g_w$) in light was significantly reduced by the deficiency in Ca and Mg, and accompanied by a lower ratio in K concentration between guard cells and epidermal cells: Kgd/Kep = 1.4 *vs.* 1.9 in controls. Decreased K accumulation in the guard cells was not expected with regard to Ca depletion in the leaves. During stomatal opening, an inward $K^+$ channel allows $K^+$ influx into the guard cell, which is activated by both plasma membrane

hyperpolarisation and low concentration in cytosolic calcium ion [12, 34, 35]. A delay in stomatal opening with light supply and a reduction in steady-state $g_w$ was also recorded for Ca-deficient seedlings of *Quercus robur* [31]. The authors hypothesized that lower photosynthesis in Ca-deficient oaks [31] and in Ca-Mg deficient beechs (this study) could have reduced the production and the mobilisation of organic osmoticum required for stomatal opening. Therefore, a depletion in malate$^{2-}$, resulting in a lower negative charge in the guard cell vacuole, may explain the reduced accumulation of $K^+$.

With excess Al in the nutrient solution, stomatal response to all stimuli was similar to that of Ca-Mg deficient beech. It is noteworthy that despite enhanced K concentration in the guard cells, $g_w$ in the dark was not significantly increased. In fact the raise in guard cell turgor, required for stomatal opening, depends on the ratio in osmoticum between the epidermal cells and the guard cells. As a result of Al-induced K increase in both cell types, Kgd/Kep remained comparable to the control. As in –CaMg plants, $g_w$ in light were lowered and accompanied by a lower Kgd/Kep: 1.4 *vs.* 1.9 in controls. Al was found to be a specific inhibitor of inward K channels in the plasmalemma of the guard cells [33], and may have limited K influx. However, the reduction in gw was not significantly higher than in –CaMg plants, and the level of Ca-Mg deficiency were similar for both treatments. It is therefore impossible to assume whether lower $g_w$ and K concentration in guard cells are a primary effect of Al toxicity or a consequence of Al-induced Ca-Mg depletion.

Beech seedlings exposed to both Al stress and a deficiency in Ca and Mg were characterized by i) an increased stomatal conductance in darkness ii) very limited stomatal movements in reponse to the different stimuli iii) a strong dysfunction of K fluxes between the guard cells and the epidermal cells. In darkness, high $g_w$ was not accompanied by increasing Kgd/Kep; and light supply promoted a slight increase of $g_w$ without any K accumulation in the guard cells. Stomatal aperture may therefore be attributed to a raise in organic compounds in the guard cell vacuole or a disturbance in cell structure. The discrepancy between stomatal response in +Al and +Al–CaMg plants was surprising as no difference could be detected in Al accumulation nor in Ca and Mg concentrations in the leaves between the two treatments. Nevertheless, this result strongly suggests the occurrence of a leaf senescence in +Al–CaMg seedlings. This hypothesis was corroborated by the presence of leaf necrosis.

With regard to photosynthesis, Hampp and Schnable [15] found that a 10 μM Al concentration caused severe damage to the membranes of isolated chloroplasts from *Spinacea oleracea*. Therefore, if Al reached the chloroplasts of intact plants, it is likely to depress the photosynthetic acivity. Beech seedlings exposed to excess Al or Ca-Mg deficiencies exhibited a reduction in net $CO_2$ assimilation rates (A) on a leaf area basis. However, on a chlorophyll concentration basis, A remained comparable to the control value for both treatments. These results suggest that the reduction in photosynthesis at leaf level could be accounted for by lowered chlorophyll content. Schlegel and Godbold [32] proposed similar conclusions for *Picea abies*. By feeding the needles of Al-stressed plants directly with Mg, they observed an increase in Mg content of the needles. As a result, both chlorophyll concentration and $CO_2$ uptake were enhanced. They postulated that Al effect on photosynthesis was not directly mediated by Al toxicity, but is the consequence of the Al-induced Mg deficiency. However, Mg fumigation also decreased the amount of Al in the leaves and therefore could have suppressed a potential direct toxicity of Al. In beech seedlings submitted to Al, the relative Mg deficiency in the leaves was comparable to that recorded with decreasing Ca-Mg nutrition. And, despite higher Al concentration in the parenchyma cells, the reduction in A was not significantly higher in +Al plants than in –CaMg plants. Calcium deficiency was also shown to reduce A for oak seedlings, without any reduction in chlorophyll content [31]. This reduction in photosynthesis was ascribed to reduced $CO_2$ avaibility in the chloroplast. In both oak [31] and beech (this study) seedlings, the constancy in the $CO_2$ mole fraction in the sub stomatal spaces ($c_i$) suggests a non stomatal limitation of $CO_2$ influx into the leaf. However, an overestimation in the computation of $c_i$, like those reported by Terashima et al. [38] in droughted plants cannot be ruled out. Additional experiments would be needed to estimate the impact of Al on $CO_2$ mole fraction at the chloroplast level, and on both stomatal and mesophyll limitations of $CO_2$ diffusion into the leaf. Finally, it is once again impossible to assume whether lower net assimilation rates is a primary effect of Al toxicity or a consequence of Al-induced Ca-Mg depletion.

With combining excess Al and a deficiency in Ca and Mg, the reduction in net $CO_2$ assimilation rates was more pronounced. Furthermore, the decrease in chlorophyll amounts could not explain the reduction in A. On a chlorophyll concentration basis, A was 50% lower than in controls. Potential Al injury on the chloroplast integrity should be investigated by means of chlorophyll a fluorescence analysis.

## 5. CONCLUSION

This study confirms that Al i) disturbs the plant nutrient balance ii) is to be considered as a complex abiotic desease and iii) that Ca avaibility plays a major role in limiting Al-induced injury.

Aluminium was shown to reduce light stomatal conductance and net carbon assimilation of beech seedlings. This reduction of stomatal aperture is the result of limited accumulation of $K^+$, and may be of organic osmoticum, in the guard cell vacuole. It is likely that such effect is the result of Al-induced deficiency in Ca.

The major finding of this study is an Al $\times$ nutrient deficiency interaction leading to a strong stomatal dysfunction and a further reduction in leaf carbon assimilation. Notably, the lack of stomatal reactivity to ABA, the endogenous signal inducing stomatal closure with soil water depletion, may facilitate drought-induced decline processes. This is of major importance with regard to potential changes in soil chemistry due to acidic anthropogenic inputs. Indeed, Weissen [46] reported a significant increase of the acidity for several forest soils of the Ardenne.

Finally, the reduced photosynthesis observed on beech seedlings may result in a loss in wood productivity.

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## REFERENCES

[1] Allan A.C., Fricker M.D., Ward J.L., Beale M.H., Trewavas A.J., Two Transduction Pathways Mediate Rapid Effects of Abscisic Acid in *Commelina* Guard Cells, Plant Cells 6 (1994) 1319-1328.

[2] Asp H., Bengtsson B., Jensén P., Growth and cation uptake in spruce (*Picea abies* Karst.) grown in sand culture at various aluminium supply, Plant Soil 111 (1988) 127-133.

[3] Balsberg Pahlsson A.M., Influence of aluminium on biomass, nutrients, soluble carbohydrates and phenols in beech (*Fagus sylvatica*), Physiol. Plant. 78 (1990) 79-84.

[4] Barnes J.D., Balaguer L., Manrique E., Elvira S., Davison A.W., A reappraisal of the use of DMSO for the extraction and determination of chlorophylls-a and chlorophylls-b in lichens and higher plants, Environ. Exp. Bot. 32 (1992) 85-100.

[5] Bengtsson B., Influence of aluminium and nitrogen on uptake and distribution of minerals in beech roots (*Fagus sylvatica*), Vegetatio 101 (1992) 35-41.

[6] Bengtsson B., Asp H., Jensen P., Berggren D., Influence of aluminium on phosphate and calcium uptake in beech (*Fagus sylvatica*) grown in nutrient solution and soil solution, Physiol. Plant. 74 (1988) 299-305.

[7] Blatt M.R., Thiel G., Trentham D.R., Reversible inactivation of $K^+$ channels of *Vicia faba* stomatal guard cells following the photolysis of caged inositol 1,4,5-triphosphate, Nature 346 (1990) 766-769.

[8] Bonneau M., Le diagnostic foliaire, Revue Forestière Française 19-28 (1988).

[9] De Silva D.L.R., Cox R.C., Hetherington A.M., Mansfield T.A., Suggested involvement of calcium and calmodulin in the responses of stomata to abscisic acid, New Phytol. 101 (1985) 555-563.

[10] DeWald L.E., Sucoff E.I., Ohno T., Buschena C.A., Response of northern red oak (*Quercus rubra* L.) seedlings to soil solution aluminium, Can. J. For. Res. 20 (1990) 331-336.

[11] Ericsson E., Göransson A., Van Oene H., Gobran G., Interactions between aluminium, calcium and magnesium - Impacts on nutrition and growth of forest trees, Ecol. Bull. 44 (1995) 191-196.

[12] Fairley-Grenot K.A., Assmann S.M., Permeation of $Ca^{2+}$ through $K^+$-selective channels in the plasma membrane of *Vicia faba* guard cells, J. Memb. Biol. 128 (1992) 103-113.

[13] Garrec J.P., Microanalyse des éléments diffusibles en biologie. in: Quintana C. and Malpern S. (Eds.), Microanalyse X en biologie, Société française de microscopie électronique, Paris (1983) pp. 141-150.

[14] Gilroy S., Fricker M.D., Read N.D., Trewavas A.J., Role of calcium in signal transduction of *Commelina* guard celles, Plant Cells 3 (1991) 333-344.

[15] Hampp R., Schnabl H., Effect of aluminium ions on $^{14}CO_2$-fixation and membrane system of isolated spinach chloroplasts, Z. Pflanzenphysiol. 76 (1975) 300-306.

[16] Hecht-Bucholz C., Jorns C.A., Keil P., Effects of excess aluminium and manganese on Norway spruce seedlings as related to magnesium nutrition, J. Plant Nutr. 10 (1987) 1103-1110.

[17] Horton B.D., Edwards J.H., Diffusion resistance rates and stomata aperture of peach seedlings as affected by aluminium concentration, Hort. Sci. 11 (1976) 591-593.

[18] Inoue H., Katoh Y., Calcium inhibits ion-stimulated stomatal opening in epidermal strips of *Commelina communis* L, J. Exp. Bot. 38 (1987) 142-149.

[19] Kearns E.V, Assmann S.M., The Guard Cell-Environment Connection, Plant Physiol. 102 (1993) 711-715.

[20] Kochian L.V., Cellular mechanisms of aluminium toxicity and resistance in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 46 (1995) 237-260.

[21] Kruger E., Sucoff E., Growth and nutrient status of *Quercus rubra* L. in response to Al and Ca, J. Exp. Bot. 40 (1989) 653-658.

[22] Laffray D., Louguet P., Garrec J.P., Microanalytical studies of potassium and chloride fluxes and stomatal movements of two species : *Vicia faba* and *Pelargonium x hortorum*, J. Exp. Bot. 33 (1982) 771-82.

[23] Lambert J., Parmentier M., Léonard C., Weissen F., Reginster P., Premiers enseignements de l'analyse des sols forestiers en région wallone, Silva Belgica 97 (1990) 7-12.

[24] Macallum A.B., On the distribution of potassium in animal and vegetable cells, J. Physiol. 32 (1905) 95-128.

[25] MacRobbie E.A.C., Ion fluxes in “isolated” guard cells of *Commelina communis* L, J. Exp. Bot. 32 (1981) 545-562.

[26] Mansfield T.A., Hetherington A.M., Atkinson C.J., Some current aspects of stomatal physiology, Annu. Rev. Plant Physiol. Plant Mol. Biol. 41 (1990) 55-75.

[27] McAinsh M.R., Brownlee C., Hetherington A.M., Abscisic acid-induced elevation of guard cell cytosolic $Ca^{2+}$ precedes stomatal closure, Nature 343 (1990) 186-188.

[28] McAinsh M.R., Brownlee C., Hetherington A.M., Calcium ions as second messengers in guard cell signal transduction. Physiol, Plant 100 (1997) 16-29.

[29] McDermitt D.K., Norman J.M., Travis J.T., Ball T.M., Arkebauer T.J., Welles J.M., Roemer S.R., $CO_2$ response curves can be measured with a field-portable closed-loop photosynthesis system, Ann. Sci. For. 46 Suppl. (1989) 416s-420s.

[30] Ridolfi M., Garrec J.P., Louguet P., Laffray D. Effects of potassium and calcium deficiencies on stomatal functioning in intact leaves of *Vicia faba* L, Can. J. Bot. 72 (1994) 1835-1842.

[31] Ridolfi M., Roupsard O., Garrec J.P., Dreyer E., Effects of a calcium deficiency on stomatal conductance and photosynthetic activity of *Quercus robur* seedlings grown on nutrient solution, Ann. Sci. For. 53 (1996) 325-335.

[32] Schlegel H., Godbold D.L., The influence of Al on the metabolism of spruce needles, Water, Air and Soil Pollution 57-58 (1991) 131-138.

[33] Schnable H., Zeiger H., Über die Wirkung von Aluminum-ionen auf die Stomatabewegung von *Vicia faba* Epidermen, Z. Pflanzenphysiol. 74 (1975) 394-403.

[34] Schroeder J.I., Hagiwara S., Cytosolic calcium regulates ion channels in the plasma membrane of *Vicia faba* guard cells, Nature 338 (1989) 427-30.

[35] Schroeder J.I., Hedrich R., Fernandez J.M., Potassium-selective single channels in guard cell protoplasts of *Vicia faba*, Nature 312 (1984) 361-362.

[36] Schwartz A., Role of $Ca^{2+}$ and EGTA on stomatal movements in *Commelina communis* L, Plant Physiol. 79 (1985) 1003-1005.

[37] Stienen H., Veränderungen in Wasserhaushalt junger Koniferen in säurer und $Al^{3+}$ haltiger Nähr und Bodenlösung, Forstarchiv. 57 (1986) 227-231.

[38] Terashima I., Wong S.C., Osmond C.B., Farquhar G.D., Characterization of non-uniform photosynthesis induced by abscisic acid in the leaves having different mesophyll anatomies, Plant Cell Physiol. 29 (1988) 385-395.

[39] Thornton F.C., Schaedle M., Raynal D.J., Tolerance of Red Oak and American and European Beech Seedlings to Aluminium, J. Environ. Qual. 18 (1989) 541-545.

[40] Trewavas A., Le Calcium, c'est la Vie : Calcium Makes Waves, Plant Physiol. 120 (1999) 1-6.

[41] Van Praag H.J., Weissen F., Sougnez-Remy S., Carletti G., Aluminium effects on spruce and beech seedlings. II Statistical analysis of sand culture experiments, Plant Soil 83 (1985) 339-356.

[42] Van Praag H.J., Weissen F., Delecour F., Ponette Q., Aluminium effects on forest stands growing on acid soil in the Ardennes (Belgium), Belg. J. Bot. 124 (1991) 128-136.

[43] Van Praag H.J., Weissen F., Dreze P., Cogneau M., Effects of aluminium on calcium and magnesium uptake and translocation by root segments of whole seedlings of Norway spruce (*Picea abies* Karst.), Plant Soil 189 (1997) 267-273.

[44] Von Caemmerer S., Farquhar G.D., Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves, Planta 153 (1981) 376-387.

[45] Weissen F., La germination des faînes conservées à basse température, Bull. Soc. Roy. For. de Belgique 87 (1980) 81-88.

[46] Weissen F., Dix ans plus tard : l'état de la question sur le dépérissement en forêt wallone, Silva Belgica 103 (1996) 9-19.

[47] Weissen F., Van Praag H.J., André P., Maréchal P., Causes du dépérissement des forêts en Ardenne : observations et expérimentation, Silva Belgica 99 (1992) 9-13.

[48] Zhao X.J., Sucoff E., Stadelmann E.J., $Al^{3+}$ and $Ca^{2+}$ alteration of membrane permeability of *Quercus rubra* cortex cells, Plant Physiol. 83 (1987) 159-162.