Physiological responses of *Lupinus luteus* to different copper concentrations

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Abstract

Yellow lupin (*Lupinus luteus* L.) plants were grown in hydroponic solution for 15 d under different copper concentrations (0.1, 0.5, 1.0, 10, 25 and 50 μM). With increasing Cu concentration total biomass was not affected, leaf area slightly decreased, while chlorophyll content decreased considerably. Cu content increased significantly both in roots and in leaves, but the contents of other ions were only slightly affected at the highest Cu concentration (Mn content decreased both in roots and in leaves, P content decreased only in leaves and Zn content increased in roots). Superoxide dismutase (SOD) activity increased up to day 7 after copper application. Peroxidase (GPOD) and polyphenol oxidase (PPO) activities also increased, while catalase (CAT) activity remained constant.

Additional key words: yellow lupin, oxidative stress, heavy metal toxicity.

Introduction

Copper is a plant micronutrient that is an essential component of several enzymes and coenzymes involved in metabolic pathways of plants. However, at high concentrations Cu can become phytotoxic affecting plant development due to direct or indirect interference with numerous physiological processes (Maksymiec 1997, Vangronsveld and Clijsters 1994). Described symptoms of copper phytotoxicity include stunted growth, leaf chlorosis (Baron *et al.* 1995, Fernandes and Henriques 1991), changes in mineral content and several enzyme activities (Brun *et al.* 2003, Maksymiec 1997, Mazhoudi *et al.* 1997, Mocquot *et al.* 1996). According to Clijsters *et al*. (1999) some of these changes may be observed before visible symptoms become evident.

One of the main effects of copper is the induction of oxidative stress, leading to the production of reactive oxygen species (ROS) that can cause the activation of different antioxidative pathways (Clijsters *et al.* 1999, Cuypers *et al.* 1999, Mazhoudi *et al.* 1997, Van Assche and Clijsters 1990). Enzymes can be activated which are implicated in the removal of superoxide (*e.g.* superoxide dismutase) and of H_2O_2 (like catalase and peroxidase) and those involved in the ascorbate-glutathione pathway (Gupta *et al.* 1999, Cuypers *et al.* 2000).

As yellow lupin seeds are used in animal feed, the possibility of plant uptake and accumulation of heavy metals in seed can be dangerous for both animal and human nutrition. However, to date, no studies of this subject have been carried out with this lupin species and copper toxicity. Brennan and Mann (2005) have shown that yellow lupins can accumulate more cadmium than other lupin species.

In order to improve our knowledge regarding the tolerance of yellow lupin plants to excess copper we studied the effect of this metal on fresh and dry matter, leaf area, chlorophyll content, contents of calcium, sodium, magnesium, potassium, phosphorus, copper, zinc, iron and manganese, and activities of guaiacol peroxidase (GPOD), catalase (CAT), superoxide dismutase (SOD) and polyphenol oxidase (PPO).

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Abbreviations: CAT - catalase; d.m. - dry mass; f.m. - fresh mass; GPOD - guaiacol peroxidase; PPO - polyphenol oxidase; SOD - superoxide dismutase.
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Materials and methods

Plants and copper treatments: Yellow lupin (*Lupinus luteus* L. cv. Cardiga) seeds were germinated in moist filter paper soaked with deionised water at a temperature above 20 ºC for 7 d. After germination, the seedlings were planted on perforated polystyrene plates, floating on an aerated Hoagland nutrient solution, which was renewed every week. The plants were grown for 6 weeks on hydroponic culture in a growth chamber at temperature of 18 - 24 ºC, relative humidity of 65 %, and 14-h photoperiod with irradiance of 250 μmol m^{-2} s^{-1}. After that, plants were treated with nutrient solutions containing different copper concentrations: 0.1 μM (control), 0.5, 1.0, 10, 25 and 50 μM, supplied as $CuSO_4$.

Growth parameters, chlorophyll content and mineral content: Leaves and roots of plants were sampled after 3, 7, 11 and 15 d of copper application, and all the determinations were repeated at least 3 times. For dry mass determination, samples were oven dried at 105 ºC until constant mass. Leaf area determination was performed using an *HP Scanjet 3400* scanner and the software *ImageJ* (*ImageJ 1.30v*, *NIH*, Maryland, USA). Chlorophyll content was determined by a non-destructive method using the *Minolta SPAD-501* (Osaka, Japan) apparatus. Results were expressed in arbitrary units (SPAD units) that are proportional to chlorophyll content (Madeira *et al.* 2000). For mineral composition determination (Ca, Cu, Fe, K, Mg, Mn, Na and Zn), samples of dried plant material were ashed at 480 ºC in a muffle furnace, twice digested in 10 cm^3 of 3 M HCl at 90 ºC and analysed by flame atomic absorption spectrophotometer (*Unicam Solaar M*, Massachusetts, USA). Phosphorus was determined by the molybdo-vanadate colorimetric method using an *Hitachi U-2000* (Tokyo, Japan) UV/Vis spectrophotometer.

Enzyme assays: Crude extract was obtained by maceration of *ca*. 0.5 g of leaves in the presence of 2 % (m/m) insoluble polyvinylpolypyrollidone (PVP), with 1 cm^3 of 100 mM Tris-HCl buffer (pH 7.8), containing 3 mM dithiothreithol (DTT) and 1 mM ethylenediamine-tetracetic acid (EDTA). The homogenate was centrifuged at 10 000 *g* for 30 min (*Sigma 3-18K*, Osterode am Harz, Germany) and the supernatant was filtered through 0.20 μm filters. All procedures were performed at a temperature below 4 ºC. Peroxidase (EC 1.11.1.7) activity was determined according to a modified Tang and Newton (2005) method. The formation of tetraguaiacol was followed for 2 min (at the wavelength of 470 nm) in a 3.5 cm^3 reaction mixture containing 30 mM 2-methoxyphenol (guaiacol) and 4 mM H_2O_2 in 0.2 M sodium acetate buffer (pH 6.0). Enzymatic activity is defined as the consumption of 1 μmol of guaiacol per min and per cm^3 at room temperature, using coefficient of absorbance for tetraguaiacol of 2.55 μM^{-1} cm^{-1}. Catalase (EC 1.11.1.6) activity was determined using a modified Aebi (1983) method, measuring the decrease in absorbance at 240 nm for 2 min, in a solution containing 10 mM of H_2O_2 in 50 mM phosphate buffer (pH 7.0). Enzymatic activity is defined as the variation in H_2O_2 absorbance per min and per cm^3 using coefficient of absorbance of 3.94 nM^{-1} cm^{-1}. Superoxide dismutase (EC 1.15.1.1) activity was measured according to a modified Rubio *et al.* (2002) method, measuring the increase in absorbance at 550 nm for 2 min in a solution containing 0.5 mM xanthine, 0.05 mM ferricytochrome *c*, 0.1 mM EDTA and xanthine oxidase in 100 mM potassium phosphate buffer (pH 7.6). Enzymatic activity is defined as the enzyme quantity needed to inhibit the reduction of ferricytochrome-C by 50 % per min and per cm^3. Polyphenol oxidase (EC 1.10.3.1) activity was measured according to the modified method of Oktay *et al.* (1995) by measuring the increase in absorbance at 420 nm for 2 min, using a reaction solution containing 30 mM catechol in 50 mM phosphate buffer (pH 7.0). Enzymatic activity is defined as the variation in catechol absorbance per min and per cm^3. All measurements were performed in triplicate with samples collected from several plants.

Statistical analysis: Statistical analysis was performed using the software *SPSS 13.0* (*SPSS Inc*, 1989-2004). The results were subjected to a one-way *ANOVA* using the Tukey test to check for significant differences between means ($P < 0.05$). Error bars in figures represent standard deviation.

Results

In the roots of plants growing at 50 μM Cu, endogenous Cu content increased 32 times compared to the control. Cu was also translocated to the leaves and its content increased 5 fold compared to the control (Fig. 1). Fresh masses of both roots and leaves were not significantly affected (results not shown) and root growth was only affected after 15 d at 25 and 50 μM Cu. At these copper concentrations darkening of the roots was also apparent. Leaf area showed only a slight decrease for 25 and 50 μM Cu in nutrient solution (results not shown). Although total biomass was not affected by these Cu concentrations, dry matter content increased with Cu concentration both in leaves and roots with significant differences at the two highest Cu concentrations (Fig. 1).

For plants growing in 50 μM of Cu symptoms of chlorosis and senescence of leaves were observed. SPAD values (which are correlated to chlorophyll content) decreased during 20-d treatment for all Cu concentrations

tested. There was a strong negative influence of both copper concentration and time (Fig. 2). The obtained results were fitted to exponential equations with good correlation ($r^2 \geq 0.94$).

The contents of Na, K, Ca, Mg and Fe remained constant both in the leaves and in the roots. Only a decrease in Mn contents, both in roots and in leaves was detected, as well as a decrease in leaf P content and an increase in root Zn uptake (Fig. 3).

The activities of peroxidase, polyphenol oxidase, superoxide dismutase mostly initially increased and then decreased at the longer treatment duration, and also mostly increased with increased Cu concentration (Fig. 4). The exception was catalase that showed no significant differences for the whole time and copper concentration range.

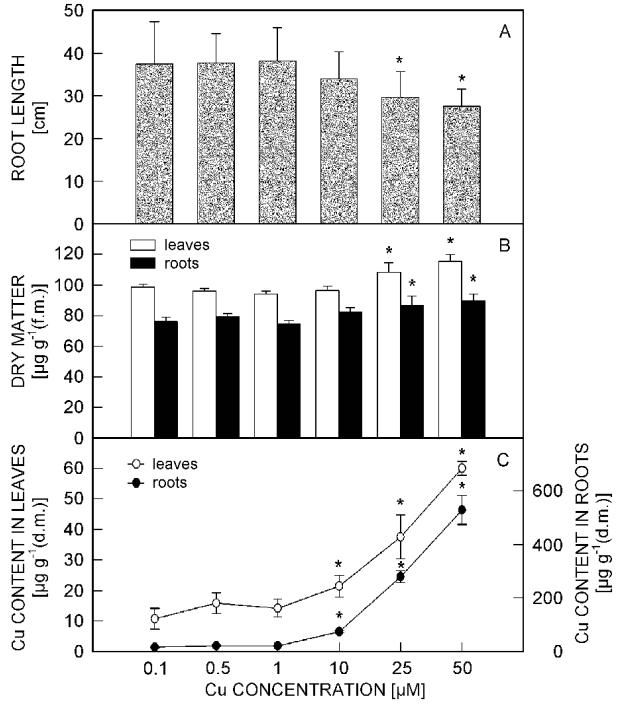

Fig. 1. Effect of different copper concentrations in nutrient solution for 15 d on root length (*A*), leaf and root dry matter content (*B*), and Cu contents in leaves and roots (*C*). Significant differences ($P < 0.05$, Tukey test) in relation to the control (0.1 μM) are indicated with an *asterisk*.

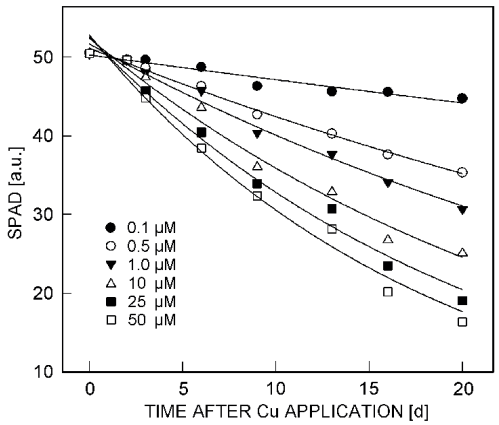

Fig. 2. Effect of Cu concentration in nutrient solution on chlorophyll content (measured as SPAD values) as a function of time. Corresponding exponential equations:
0.1 μM, $y = 0.268e^{-0.0065x}$, $r^2 = 0.938$
0.5 μM, $y = 51.129e^{-0.0186x}$, $r^2 = 0.994$
1.0 μM, $y = 51.650e^{-0.0254x}$, $r^2 = 0.988$
10 μM, $y = 52.381e^{-0.0378x}$, $r^2 = 0.980$
25 μM, $y = 52.552e^{-0.0471x}$, $r^2 = 0.982$
50 μM, $y = 52.798e^{-0.0547x}$, $r^2 = 0.982$

Discussion

Enzyme activities were monitored every 3 - 4 d in yellow lupin plants growing in hydroponic solution containing 0.1 (the control), 0.5, 1, 10, 25 and 50 μM Cu SO_4 for 15 d. No toxic effects were detected for plants grown in 0.5 and 1.0 μM Cu (except for a small decrease in chlorophyll content) and at 1.0 μM Cu, which is 10 times the control value, Cu contents in both roots and leaves increased only 1.3 fold compared to the control. Most of the Cu absorbed by the plant remained, as expected, in the roots. Translocation to the upper plant parts also occurred, mainly in the plants growing in 25 and 50 μM Cu, where the amount of Cu in the leaves was several times higher compared to the control and 13 and 11 % of that in the roots, respectively. Proportionally, the increase in Cu content in roots was much higher than in leaves. During this treatment plant growth was only affected at 50 μM Cu, which can be considered toxic for most plant species. At this Cu concentration some chlorosis and senescence of leaves was visible. Leaf expansion, a common target for toxic metals, was only slightly affected in our study and thus Cu contents in the shoots were not high enough to affect leaf expansion significantly. Potassium, which is an important element for leaf expansion, remained at a constant level in the leaves of yellow lupin during this experiment. Cuypers *et al.* (2000) reported that excess Cu significantly reduced leaf area of *Phaseolus vulgaris* although the effect on shoots growth was less evident.

The decrease in chlorophyll content (as shown by SPAD values) indicated that the photosynthetic system was affected and this is a frequently described effect of Cu toxicity (Liu *et al.* 2004). In heavy metal studies, it is sometimes difficult to differentiate between the direct effect of the metal and indirect effects. For example, a decrease in iron in the leaves has been reported as the consequence of heavy metal toxicity leading to a general decrease in photosynthetic capacity (Agrawal and Sharma 2006, Patsikka *et al.* 2002). In our case, iron content

remained constant in the whole experiment and thus it is not the cause for the observed chlorophyll decrease, which then could be due to inhibition of chlorophyll synthesis (Fernandes and Henriques 1991) or Cu-induced chlorophyll degradation (Liu *et al.* 2004, Prasad *et al.* 2001). The observed decrease in chlorophyll content with time was well described by exponential equation. This could indicate that chlorophyll or chloroplast destruction follow a first order kinetics.

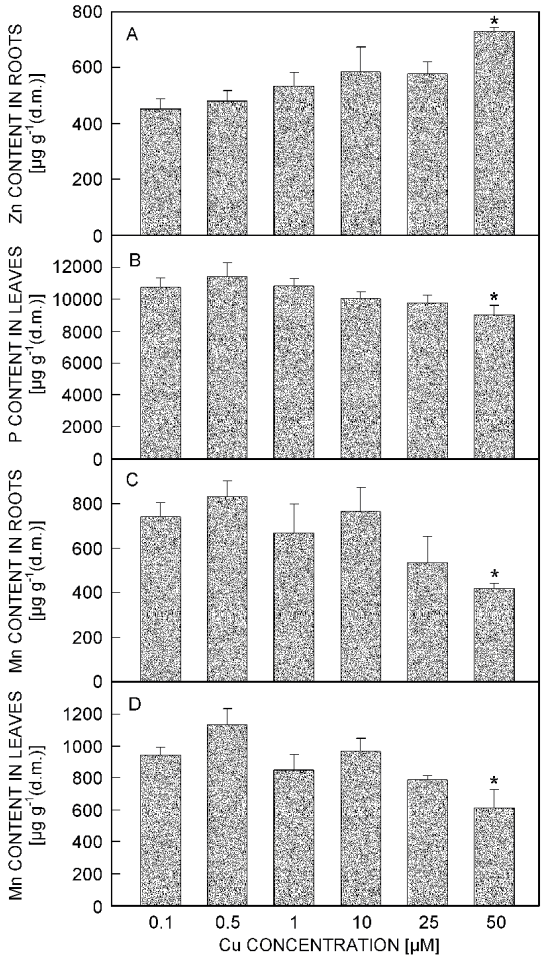

Fig. 3. Contents of selected minerals as a function of Cu concentration in nutrient solution, measured after 15 d of growth. *A* - Zn in roots, *B* - P in leaves, *C* - Mn in roots, *D* - Mn in leaves. Significant differences ($P < 0.05$, Tukey test) in relation to the control (0.1 μM) are indicated with an *asterisk*.

The roots are in direct contact with the nutrient solution and are therefore the first target for toxic effects. Our results show that Cu was mainly sequestered in the roots and that root growth was affected at 25 and 50 μM Cu, this being a commonly described effect of toxic concentrations of Cu (Jiang *et al.* 2001, Wojcik and Tukiendorf 2003). As Cu can affect membrane integrity, root water content was reduced affecting pressure potential and growth (Jouili and Ferjani 2003).

Mineral nutrition can also be affected by Cu toxicity (Alaoui-Sosse *et al.* 2004, Österås and Greger 2006, Ouzounidou *et al.* 1995) but it varies according to plant species and experimental conditions (Mocquot *et al.* 1996). In our study, the mineral contents remained remarkably stable both in roots and in leaves, with a small increase of Zn only in the roots, a small decrease of P in the leaves and a decrease of Mn in both roots and leaves. It thus seems that root Mn uptake system and Mn translocation was affected by Cu excess. However, as Mn transporters are thought to be linked to other nutrients (Grotz and Guerinot 2006), and these were not affected in our study, the decrease in Mn levels is probably not due to direct Cu action on these transporters. This could be a result of other antagonistic effects of Cu on Mn like a nonspecific effect on Mn absorption/translocation due to affected root function as proposed by Kopittke and Menzies (2006). Similar effects have been described for Cu toxicity in rice (Lidon and Henriques 1992).

To evaluate the plant defence against the potential oxidative stress imposed by Cu, the activities of several antioxidative enzymes was studied; namely, GPOD, CAT, PPO and SOD. SOD is in the first line of defence against oxidative stress and our results show a transient increase in SOD enzyme activity in early periods of Cu toxicity (3 and 7 d). After that the SOD activity decreased to control levels. This shows that the increase in its activity was probably related to a defensive mechanism against oxidative stress and not to other factors, as Mn decreased, Fe and Zn remained constant and only Cu increased. The decrease in SOD activity after the 7th day probably indicates that enzyme expression is being directly affected by the induced Cu toxicity. Two enzymes usually involved in quenching H_2O_2 are catalase and peroxidase. Our results show that GPOD activity increases constantly both with time and with Cu concentration in nutrient solution but CAT activity was not significantly affected. It does seem that GPOD is involved in H_2O_2 elimination in yellow lupin species while CAT is not. It has been reported that when CAT activity is reduced, the activity of other ROS-scavenging enzymes increases as a compensatory mechanism (Gratão *et al.* 2005) and this could be the case for the observed increase in GPOD activity. This, together with the increase in SOD activity, is thus indicative of the early activation of antioxidative defence mechanisms. In fact, the role of GPOD and CAT in plants subjected to excess Cu seems to be highly dependant on plant species (Gratão *et al.* 2005, Panda 2008). Jouili and Ferjani (2003) and Tewari *et al.* (2006) reported an increase in CAT and GPOD activity in sunflower roots and mulberry plants, respectively, while Chatterjee and Chatterjee (2000) reported a decrease of CAT in cauliflower leaves. Rucinska *et al.* (1999) studied the effect of lead on yellow lupin roots and concluded that GPOD activity increased both with time and lead concentrations while the activity of SOD and CAT reached a peak and then decreased. This reinforces our conclusion that GPOD and SOD have a predominant role in the antioxidative response of this lupin species.

PPO is a ubiquitous Cu-containing enzyme whose function in plants remains to be fully explained (Mayer 2006) but is known to be involved in plant defence against pathogens and different stresses. Our results show an increase in the activity of this enzyme but whether this is due to a putative action in relation to Cu induced stress or to the increase in plant Cu contents remains unclear. In a previous study (Martins and Mourato 2006) we observed an increase in this enzyme activity with Cu in tomato plants and a possible H_2O_2-quenching role was hypothesised. Other authors have suggested the involvement of PPO in response to Cu stress in *Panax ginseng* roots (Ali *et al*. 2006) or in lignification changes in relation to heavy metal stress in *Silene paradoxa* (Gonnelli *et al.* 2001).

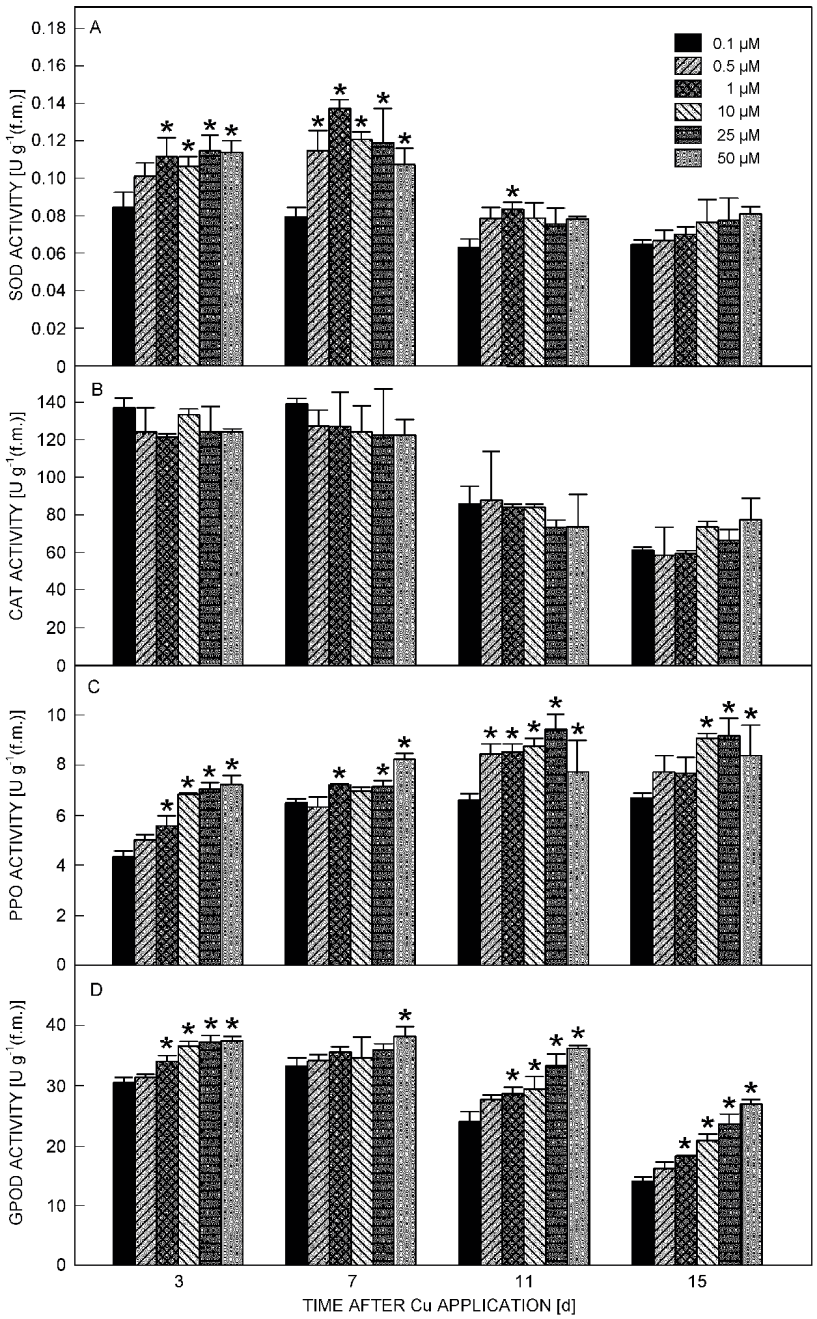

Fig. 4. Enzyme activities in leaves as a function of Cu concentration in nutrient solution and time. *A* - superoxide dismutase, *B* - catalase, *C* - polyphenol oxidase, *D* - guaiacol peroxidase. Significant differences ($P < 0.05$, Tukey test) in relation to the control (0.1 μM) are indicated with an *asterisk*.

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La Croix, I.F.: **The New Encyclopedia of Orchids. 1500 Species in Cultivation. -** Timber Press, Portland - London 2008. 524 pp. USD 59.95. ISBN-13: 978-0-88192-876-1.

This Encyclopedia has been written with the aim to reflect the latest scientific research in orchid DNAs the results of which have led to a radical reassessment of orchid classification. Long-accepted names of both the species and genera have been changed. With an estimated 25 000 orchid species (and over 100 000 hybrids which are not included in the Encyclopedia) a rather rigorous selection was necessary. The first four short chapters are an introduction to orchids as plants, their cultivation, pests and diseases, and conservation and propagation. The main part of the Encyclopedia is a detailed description of 1500 cultivated species in 350 genera presented in alphabethic order. Each item includes a description of plant morphology and sizes, characteristics of the whole plant, leaves, flowers, inflorescences, *etc*. including taxonomical and etymological data and synonyms if present. Further details concern growth and native habitat of the species as well as the country of origin and valuable remarks and tips to cultivation of the plants. An important help in the identification of the species are more than 1000 excellent photographs made by Manuel Aubron. The author of the book is Isobyl La Croix, botanist at the University of Edinburgh. She has done extensive fieldwork throughout the world, but especially in Africa, where she lived for 22 years. She has written several scientific books and articles on orchids. At the end of the book a glossary of important botanical terms, a list of References, an Index of common names and another one of scientific names of the orchid plants mentioned in the Encyclopedia are added.

The broad and deep knowledge of orchids and long-lasting experience in orchid cultivation reflected in the Encyclopedia are the guarantee for it will be used by scientists, collectors or people starting with orchid cultivation.

I. TICHÁ (*Prague*)