FOLIAR AND ROOT Cu SUPPLY AFFECT DIFFERENTLY Fe AND Zn UPTAKE AND PHOTOSYNTHETIC ACTIVITY IN SOYBEAN PLANTS

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Keywords: copper, cell suspension iron, plant, photosynthesis, soybean, uptake, zinc.

ABSTRACT

A comparative study on the effect of excess copper (Cu) supplied through leaves and root treatments in soybean plants is presented. Although Cu was accumulated in leaves upon both treatments, important differences were observed between Cu treatment of leaves and Cu supplementation in hydroponic medium. The application of 50 μM $CuSO_4$ on leaves increased chlorophyll content, oxygen evolution activity of thylakoids and fluorescence emission at 685 nm and 695 nm with respect to emission at 735 nm compared with control plants. In contrast soybean plants grown in hydroponic medium supplemented with 50 μM $CuSO_4$ exhibited lower chlorophyll content, decreased oxygen evolution activity of thylakoids and decreased fluorescence emission at 685 nm and 695 nm compared with control plants. On the other hand, the elemental analysis of leaves showed differences between the two treatments investigated. The results indicated that no antagonist interaction between Cu- and Fe-uptake took place in leaves from plants where excess Cu was applied on leaves but Cu compete with Fe-uptake in plants grown with excess Cu in the hydroponic medium. Furthermore, differences in Zn-uptake were also observed. Zn content decreased upon Cu treatment of leaves whereas the opposite was observed upon Cu treatment through roots. Interestingly, plants with Cu-treated leaves behaved similarly as cell suspensions grown in the presence of excess Cu. The results strongly indicate that different Cu-uptake and transport pathways must operate in leaf cells compared with root cells.

INTRODUCTION

Plants normally take up nutrients from soils and sediments through their roots although nutrients can be also supplied to plants as fertilizers by foliar sprays. In that case nutrients are absorbed through leaves. It is known that accumulation of certain micronutrients in soils can be toxic for plants. In particular, copper (Cu), an essential micronutrient, can be toxic in excess for most plants with the exception of a few plant species that can hyperaccumulate metals. Accumulation of Cu in soils resulting from the use of fertilizers, the application of pig and poultry slurries rich in Cu, fungicides, atmospheric deposition from industrial and urban activities, metaliferous mining or metal processing inhibit plant growth and development (Prasad and Strzałka, 1999; Kabata-Pendias and Pendias, 2001). Additionally, leaf uptake of atmospheric heavy metal emission has also been identified as an important pathway of heavy metal contamination in plants (Friedland, 1990; Salim et al., 1992). Plants grown in the presence of high levels of Cu (> 20 μg g^{-1} dry weight) show reduced biomass and chlorotic symptoms. Cu has capacity to initiate oxidative damage and interfere with important cellular functions such as photosynthesis, pigment synthesis, plasma membrane permeability and other metabolic disturbances that are responsible for a strong inhibition of plant development. Lower chlorophyll (Chl) content, alterations of chloroplast ultrastructure and thylakoid membrane composition and inhibition of photosynthetic activity have been found in leaves in such growth conditions (for reviews see Droppa and Horváth, 1990; Barón et al., 1995; Yruela et al., 2005). On the other hand, it has also been reported that Cu treatments of leaves increase Chl content and photosynthetic activity (Nagler 1973; Jasiewicz, 1981; Tong et al., 1995). The question arises as whether these different observations found in the literature are due to differences among plants or is the response to a more general

phenomenon. Acquisition of heavy metals by plants is a complex phenomenon, which involves several steps. At the root level this process includes metal transport across the plasma membrane of root cells, xylem loading and translocation, detoxification and sequestration of metal at cellular and whole plant levels. In leaves, the metal is absorbed by the epidermis, transported across the plasma membrane of cells and immobilized within the cells.

In the present work we compare the effects of an excess Cu supplementation in the hydroponic medium and a direct Cu treatment of leaves on micronutrient uptake, plant growth, and photosynthetic activity. Very few comparative studies have been conducted in this respect using the same plant variety. For that we carried out a comparative study using hydroponically grown soybean plants treated with different amounts of Cu supplementation in the growth medium or added to the leaf surface. We also used soybean cell suspensions as confirmatory experiments. The results indicated that the effects were different depending on which part of the plant was directly exposed to excess Cu.

MATERIALS AND METHODS

***Plant material and copper treatment*.-** Soybean plants (*Glycine max* cv. Volaina) were grown hydroponically in a growth chamber in half-Hoagland nutrient solution (Arnon, 1950) under 200 ± 20 μE m^{-2} s^{-1} from fluorescent and incandescent lamps at 24ºC, 70% humidity and a 16-h photoperiod. Copper treatment was made by adding to the nutrient solution 10 and 50 μM $CuSO_4$. Control medium corresponded to 0.12 μM $CuSO_4$. The hydroponic medium was changed once a week. Copper treatment on young leaves was done once a day every 4 days during the growth period with 10 and 50 μM $CuSO_4$ solutions.

***Cell suspension growth conditions*.-** Photosynthetic cell suspensions from soybean (G*lycine max* var. Corsoy) SB-P line were grown as described by Rogers et al. (1987) with some modifications. These cell suspensions were established in our laboratory in 1990. Cell suspensions were grown photomixotrophically in liquid cultures under continuous low light (30 ± 5 μE m^{-2} s^{-1}) (Bernal et al., 2006). To assay the Cu effect on cell growth the media were supplemented with 10 and 20 μM $CuSO_4$. The control medium corresponded to 0.1 μM $CuSO_4$. Cell suspensions were grown under an atmosphere with 5% CO_2 at 24ºC on a rotatory shaker (TEQ, model OSFT-LS-R) at 110 rpm in 125 ml flasks filled up to 50 ml and illuminated with cool-white fluorescent lamps (Alfonso et al., 1996; Bernal et al., 2006). Cells used for physiological and biochemical experiments were collected from cultures after 18-21 days of growth.

Isolation of thylakoid membranes.- Soybean leaves from 23-day-old plants were collected, washed twice with 2 mM EDTA solution and once with distilled H_2O, and cut into pieces. The leaf pieces were ground in buffer containing 400 mM NaCl, 2 mM $MgCl_2$, 0.2% (w/v) bovine serum albumen (BSA), 40 mM ascorbate, and 20 mM Tricine, pH 8.0 at a leaves to buffer ratio of 1:2 (w/v). The mixture was filtered through a layer of Miracloth (Calbiochem) and centrifuged at 300 x g for 2 min. The supernatant was centrifuged at 13,000 x g for 10 min and the resultant sediment resuspended in buffer containing 150 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tricine, pH 8.0 and centrifuged at 13,000 x g for 10 min. The pellet (thylakoids) was resuspended in buffer containing 400 mM sucrose, 15 mM NaCl, 5 mM $MgCl_2$, and 50 mM 2-(N-morpholino)ethanesulfonic acid (MES)-NaOH, pH 6.0 and the supernatant fractions were concentrated.

Soybean cells from 18-day-old cultures were collected, filtered through a layer of Miracloth and weighted. Cells were then resuspended in buffer containing 400 mM NaCl, 2 mM $MgCl_2$, 0.2% (w/v) sucrose, and 20 mM Tricine, pH 8.0 at a cell to buffer ratio of 1:2 (w/v), and broken with a teflon homogeneizer during 10 min with 2 min delay every 2 min homogeneization to avoid sample heating at 4ºC in darkness. Broken cells were gently stirred for 10 min and centrifuged at 300 x g for 2 min. The following steps were the same as described for soybean leaves.

The supernatants were concentrated in Centripep-10 tubes (Amicon). The thylakoid fraction was frozen in liquid nitrogen and stored at -80ºC. Chlorophyll determination was done as described by Arnon et al. (1949).

Oxygen evolution activity.- Oxygen evolution activity was measured with a Clark-type oxygen electrode at 23ºC. The light intensity on the surface of the cuvette was

3,000 μE m^{-2} s^{-1}. Thylakoids (10 μg Chl ml^{-1}) were diluted in 3 ml buffer containing 10 mM NaCl, 300 mM sucrose, and 25 mM MES-NaOH, pH 6.5. Thylakoid activities were measured in the presence of 0.5 mM 2,6-dichlorobenzoquinone (DCBQ) as artificial electron acceptor. DCBQ was dissolved in ethanol.

Determination of micronutrient elements.- Cells from 20-day-old suspensions and leaves from 23-day-old plants were washed twice with 3 mM EDTA and once with distilled H_2O to remove free cations. After washing, cells were filtered through a layer of Miracloth and dried in a ventilated owen at 60ºC for 48 h. Dried samples were treated using a standard procedure (Abadía et al., 1985). Analyses were performed in an atomic absorption spectrometer (UNICAN 969).

Fluorescence measurements.- Thylakoid membranes (20 μg Chl mL^{-1}) were resuspended in 400 mM sucrose, 20 mM NaCl, 5 mM $MgCl_2$, and 50 mM MES-NaOH, pH 6.0, placed in a 3-mm quartz tube and frozen in liquid nitrogen. Fluorescence spectra were obtained at 77 K by exciting the samples with a 1,000 W ORIEL 66187 tungsten halogen lamp and a double 0.22 m SPEX 1680B monochromator. Excitation was carried out at 470 nm. Fluorescence was detected through a 0.5 JARREL-ASH monochromator with a Hamamatsu R928 photomultiplier tube. All the measurements were corrected from the system response. The spectral linewidths (FWHM) for the excitation and the emission were 3.6 nm and 1.92 nm, respectively.

RESULTS

Effect of Cu treatments on soybean plants

Soybean plants were exposed to excess Cu (10 μM and 50 μM $CuSO_4$) during the growth at two levels: *i)* Cu was applied directly on leaves; *ii)* Cu was added to the hydroponic medium (for more details see Materials and Methods). Plants had different behaviour upon these two treatments. Plants with Cu-treated leaves showed similar growth even with 50 μM Cu(II) (Fig. 1). All plants reached similar biomass after a month of growth. A different pattern was observed in plants treated by supplementing the growth medium with excess Cu. In this case a 80% reduction of biomass was observed upon supplementation the hydroponic medium with 50 μM Cu(II) and a strong inhibition of the root development took place (Fig. 1, inset).

The results showed that Cu-treatment of leaves caused a Chl content increased by 35% and 60% upon 10 μM and 50 μM Cu-treatment, respectively. A 10% and 23% stimulation of the oxygen evolution activity in thylakoids isolated from those leaves was also found upon 10 μM and 50 μM Cu-treatment, respectively (Table I). On the other hand, fluorescence spectra at 77K with 470 nm excitation showed that F_{735}/F_{685} and F_{735}/F_{695} ratios decreased in thylakoids from Cu-treated leaves (Fig. 2, Table I). Fluorescence spectra at 77 K are often used to monitor ultrastructural changes in thylakoid membranes as a response to environmental condition variations. Thus F_{735}/F_{685} and F_{735}/F_{695} ratios can be used as a probe for the amount of antenna Chls connected to each photosystem (van Dorssen et al., 1987; Alfonso et al., 1994). The antenna Chls of photosystem I emit maximum fluorescence at 735 nm whereas those of LHCII surrounding PSII emit at 680 nm. CP43 antenna complex and PSII reaction center complexes contribute to 685 nm fluorescence maximum, and the band at 695 nm is associated with CP47 antenna complex.

Different effects were observed in plants grown in hydroponic medium supplemented with the same excess Cu. Plants exposed to 50 μM $CuSO_4$ in the hydroponic medium exhibited: *i)* a 2 fold Chl content reduction; *ii)* about 18% oxygen evolution activity decrease in thylakoids isolated from those plants; *iii)* F_{735}/F_{685} and F_{735}/F_{695} ratios increase.

Effect of Cu treatments on soybean cell suspensions

The influence of 10 μM and 20 μM $CuSO_4$ on soybean photosynthetic cell suspension was investigated. Addition of excess Cu to the control media that already contained a sufficient amount of Cu for culture growth did not reduce cell growth rate under control light illumination regime (65±5 μE m^{-2} s^{-1}), but indeed stimulated growth at limiting light conditions (30±5 μE m^{-2} s^{-1}) being the doubling time of about 4-5 days compared to 8-9 days of control suspensions (for more details see Bernal et al. 2006). Soybean Cu-treated cells exposed to 10 μM and 20 μM $CuSO_4$ in the medium after 20 days exhibited: *i)* about 23% Chl content increase; *ii)* about 25% oxygen evolution activity increase in thylakoids isolated from those cells; *iii)* F_{735}/F_{685} and F_{735}/F_{695} ratios decrease (Table I, Fig. 2). These parameters showed the same trend as in plants with Cu treated leaves. Cu concentration of 20 μM was used instead that of 50 μM because soybean cells exposed to 50 μM Cu(II) did not show such growth stimulation (Bernal et al., 2006)

Cu-uptake and its effect on other micronutrients

Different elemental composition of leaves was found in plants with Cu-treated leaves and plants grown in hydroponic medium supplemented with excess Cu. Although leaves from plants treated with 10 μM and 50 μM Cu(II) by either leaf

treatment or hydroponic medium accumulated on the average 2-3 fold higher Cu concentration when compared with control plants they differed in Fe and Zn content (Table II). We found that Fe- and Zn-uptake was affected in the same and opposite direction, respectively. In plants with 50 μM Cu treated leaves the concentration of Fe increased on the average 1.4 fold whereas their Zn content was on the average 1.2 fold lower. The opposite was observed in plants grown in hydroponic medium supplemented with 50 μM $CuSO_4$ where the concentration of Fe decreased on the average 2.5 fold whereas Zn content was on the average 1.2 fold higher compared with the control. These elemental content variations are in the same order of magnitude than those found as consequence of Fe deficiency or Cu treatments in whole plants (Pätsikkä et al., 2002; Chen et al., 2004; Rombolà, et al., 2005).

Interestingly, the same trend as in plants with Cu treated leaves was found in soybean cell suspensions. In cells grown in medium supplemented with 50 μM $CuSO_4$ the accumulation of Cu was accompanied by on the average 1.4 fold Fe increase whereas Zn content was on the average 2.0 fold lower than the control.

DISCUSSION

The results presented in this paper demonstrate that toxic concentrations of Cu can affect plants differently depending on what part of the plant is directly exposed to the element. In that sense, we observed that soybean plants behaved differently depending whether Cu supplementation was done through leaves or roots. Thus, soybean plants treated with 50 μM Cu through roots were susceptible to reduce their biomass and exhibited decreased Chl content in leaves as well as lower oxygen evolution activity in thylakoids obtained from those leaves. These findings contrasted with the Cu treatment through leaves using the same Cu concentration. A higher fluorescence emission at 685 nm and 695 nm with respect to emission at 735 nm was also observed compared with control plants. These findings are in agreement with data reported in the literature where micromolar concentrations of Cu(II) in the range of 10 to 50 μM in the growth medium decreased the growth and inhibited the photosynthetic activity (Barón et al., 1995; Yruela et al., 2005). In the second case, no apparent changes in plant growth were observed and an increase of Chl content in leaves, a higher oxygen evolution activity in thylakoids and lower fluorescence emission at 685 nm and 695 nm compared with control plants were found. The results showed that the Cu treatment through leaves stimulates Chl synthesis and photosynthetic activity. This behaviour contrasts with that normally observed in other photosynthetic organisms such as algae and plants exposed to excess Cu (Stauber and Florence, 1987; Franklin et al., 2002; Pätsikkä et al., 2002).

Our results clearly indicate that plants treated with excess Cu through leaves behave differently than plants treated by supplementing the growth medium with excess Cu. The different plant response observed upon these two Cu-treatments might be rationalized assuming different Cu-uptake strategies in leaf and root cells.

Differences in Cu transport among, cyanobacteria, algae and whole plants have also been found (La Fontaine et al., 2002). Interestingly, Cu accumulation was accompanied by Fe increase and Zn decrease in leaves treated with Cu but the opposite was observed in leaves of plants treated by supplementing the growth medium with excess Cu. Interestingly, Cu accumulation induced a Fe-uptake increase and a Zn-uptake decrease in our soybean cell suspensions, similarly to that observed in plants with leaves treated with excess Cu.

Cation homeostasis is a very complex process. In plants relatively little is known about Cu transport into and within cells showing a dependence on Cu for Fe assimilation (for reviews see Fox and Guerinot 1998; Himelblau, 2000). It has been reported that Cu and Fe compete in ion-uptake (Schmidt 1999). Pätsikkä et al. (2002) observed that excess Cu in hydroponic medium induced a Fe-deficiency in bean plants. Chen et al. (2004) observed that Fe-deficiency induces Cu accumulation in *Commelina communis* plants. Furthermore, Rombolà et al. (2005) found that Fe-deficiency increased the Cu content and decrease the Zn content in leaf blades of sugar beet grown hydroponically. However, other organisms such as mammal cells, yeast or certain algae do not appear to manifest such a competition showing a dependence on Cu for Fe assimilation (Franklin et al., 2002). A copper dependent iron assimilation pathway has been found in the unicellular green alga *Chlamydomonas reinhardtii* (La Fontaine et al., 2002). Additionally, an antagonist interaction between Cu and Zn was also observed in this alga (Herbik et al., 2002). The same scenario seems to appear in our soybean cell suspensions (Bernal et al., 2006). Thus, all the above considerations indicate that Cu-uptake strategies differ in root cells compared with leaf cells.

ACKNOWLEDGEMENTS

The authors thank M.V. Ramiro for her helpful technical assistance. M. Bernal is recipient of a predoctoral fellowship from Consejo Superior de Investigaciones Científicas (I3P Programme financed by the European Social Fund). The SB-P line was kindly provided by Prof. Jack M. Widholm (Department of Agronomy, University of Illinois at Urbana, Urbana IL). This work was supported by the Aragón Government (Grant P015/2001) to I.Y. and the Ministry of Science and Technology of Spain (BMC2002-00031) to R.P.

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FIGURE CAPTIONS

Figure 1.- Effect of excess Cu on soybean plants. Plants were treated with Cu at two levels: a) young leaves were painted once every four day period with 10 μM and 50 μM $CuSO_4$ solutions (upper panel); *b)* hydroponic medium was supplemented with 10 μM and 50 μM $CuSO_4$ (lower panel). Leaves were collected after a month of growth. For more details see Materials and Methods.

Figure 2.- Fluorescence emission spectra at 77K of thylakoid membranes from (A) leaves treated with Cu; (B) soybean plants grown in hydroponic medium supplemented with Cu; (C) Cu-treated soybean cells. (1) Control; (2) 10 μM $CuSO_4$; (3) 20 μM (C) or 50 μM $CuSO_4$ (A,B). Excitation wavelength was at 470 nm (for more details see Materials and Methods). Spectra were normalized at 739 nm (PSI emission band).

TABLE I

Photosynthetic parameters of thylakoids from soybean plant leaves and soybean cell suspensions[1]

Samples	Chlorophyll (mg g^{-1} dry weight)	Oxygen evolution (μmol O_2 mg^{-1} Chl h^{-1})	Chl *a*/Chl *b*	F_{735}/F_{685}	F_{735}/F_{695}
Soybean plant leaves					
Leaves treatment					
Control	4.6 ± 1.3	238 ± 12	3.5± 0.1	4.7± 0.2	4.8± 0.1
10 μM Cu(II)	6.3 ± 1.3	252 ± 15	3.5± 0.1	4.1± 0.1	4.2± 0.2
50 μM Cu(II)	7.5 ± 1.3	285 ± 12	3.5± 0.1	3.9± 0.0	3.8± 0.1
Hydroponic treatment					
Control	4.4 ± 1.3	242 ± 12	3.4± 0.1	4.4± 0.1	4.8± 0.1
10 μM Cu(II)	3.8 ± 0.3	220 ± 4	3.4± 0.1	5.4± 0.1	5.5± 0.2
50 μM Cu(II)	2.1 ± 0.1	197 ± 8	3.2± 0.1	5.6± 0.2	5.9± 0.1
Soybean cell suspension					
Control	3.16 ± 0.5	189 ± 7	3.1± 0.1	3.1± 0.1	3.0± 0.1
10 μM Cu(II)	3.90 ± 0.6	245 ± 11	3.1± 0.1	2.4± 0.1	2.5± 0.1
20 μM Cu(II)	3.96 ± 1.1	250 ± 14	3.1± 0.1	2.3± 0.1	2.4± 0.1

[1]Soybean plants were treated with excess Cu at two levels, *a)* young leaves were painted once a day every four day during the growth period with 10 μM and 50 μM $CuSO_4$ solutions; *b)* hydroponic medium was supplemented with 10 μM and 50 μM $CuSO_4$; media were changed weekly. Leaves were collected after a month of growth. Soybean cell cultures were grown in media supplemented with 10 μM and 20

μM $CuSO_4$. Cells were analysed after 21 days of growth. Data were obtained from three independent experiments. Values represent means ± SE (n=3).

TABLE II

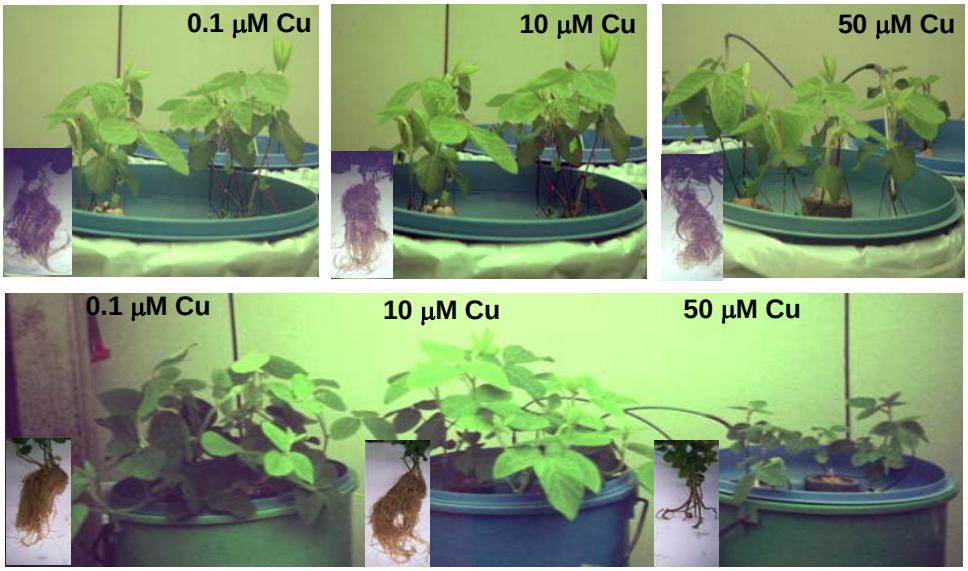
Micronutrient content [1] in plant leaves and cell suspensions [2]

Samples	Cu	Fe	Zn
Soybean plant leaves			
Control	6.8 ± 1.8	90.2 ± 1.3	90.8 ± 1.3
Foliar treatment			
10 μM Cu(II)	11.5 ± 1.3	100.7 ± 1.2	85.1 ± 1.3
50 μM Cu(II)	13.5 ± 2.3	126.8 ± 4.3	77.7± 3.3
Hydroponic treatment			
50 μM Cu(II)	23.1 ± 2.3	38.2 ± 1.7	105.6± 2.4
Soybean cell suspensions			
Control	5.9 ± 1.6	282.2 ± 7.0	351.1 ± 8.3
10 μM Cu(II)	209.5 ± 5.3	328.1 ± 4.3	161.3 ± 4.3
20 μM Cu(II)	351.2 ± 7.5	389.8 ± 3.9	174.3 ± 3.2

[1] $\mu g\ g^{-1}$ dry weight

[2] Soybean plants were treated with excess Cu at two levels, *a)* young leaves were painted once a day every four day during the growth period with 10 μM and 50 μM $CuSO_4$ solutions; *b)* hydroponic medium was supplemented with 10 μM and 50 μM $CuSO_4$; media were changed weekly. Leaves were collected after a month of growth. Soybean cell cultures were grown in media supplemented with 10 μM and 20 μM

$CuSO_4$. Cells were analysed after 21 days of growth. Data were obtained from three independent experiments. Values represent means ± SE (n=3).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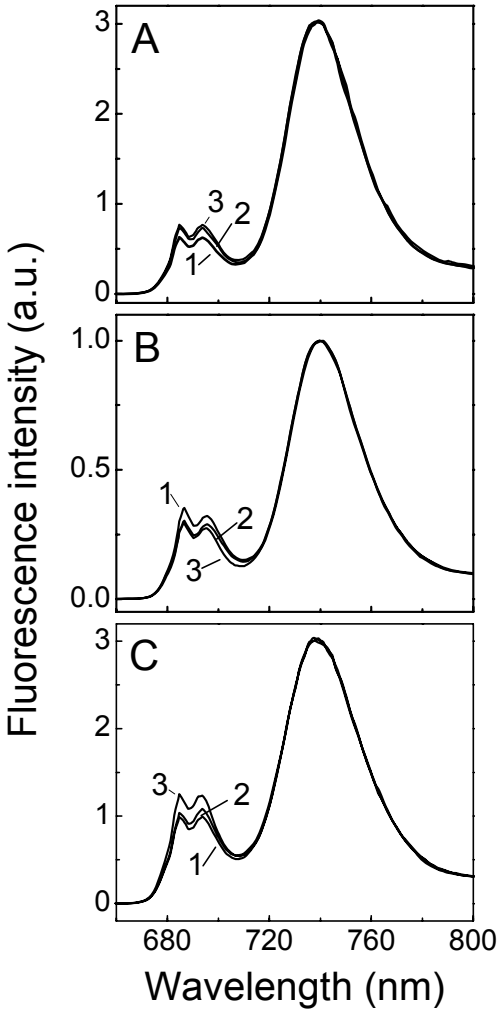
A
B
C
1
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Fluorescence intensity (a.u.)
Wavelength (nm)
680
720
760
800

Fig.2