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# A glycoconjugate from corms of saffron plant (*Crocus sativus* L.) inhibits root growth and affects *in vitro* cell viability

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## Abstract

**A glycoconjugate has been characterized from saffron corms (*Crocus sativus* L.) that inhibits the growth of roots of *Nicotiana tabacum* and *Arabidopsis thaliana*, at concentrations ranging from 1–100 μg $m^{-3}$. Roots of seedlings grown in the presence 0.1 μg $m^{-3}$ glycoconjugate showed bulging of epidermal cells, whereas at 10 μg $m^{-3}$, roots were completely devoid of hairs. At 100 μg $m^{-3}$ glycoconjugate the cell walls of the root vascular tissues were thicker and, overall, the vascular tissue was enlarged. In addition, this glycan is cytotoxic to isolated tobacco cells and protoplasts, with 50% cell death induced by 0.5 and 2μg $m^{-3}$ glycoconjugate, respectively. Morphological and biochemical changes induced by the exposure to the glycoconjugate included cell size decrease, loss of regular cell shape, cytoplasm collapse, and release of intracellular proteins. This molecule at low concentrations (0.1μg $m^{-3}$) mimics the effects of Yariv phenylglycosides and of mutant *Arabidopsis* which present defective or missing arabinogalactan-proteins (AGPs) in roots, indicating the glycoconjugate might interact with cell surface AGPs.**

Key words: *Crocus sativus* L., glycoconjugate, proteoglycan, root morphogenesis.

## Introduction

Plant proteoglycans occur abundantly in plant secretions and at cell surfaces. AGPs are a major class of plant proteoglycans (Clarke *et al*., 1979; Fincher *et al*., 1983; Chasan, 1994; Du *et al*., 1996*a*; Knox, 1995; Nothnagel, 1997). The physiological functions of the AGPs are not established, although roles as lubricants, nutritive factors, components of cell recognition processes during plant morphogenesis, and factors influencing somatic embryogenesis have been suggested (Clarke *et al*., 1979; Fincher *et al*., 1983; Kreuger and van Holst, 1993; Chasan, 1994). Cell surface AGPs have been implicated in cell proliferation (Serpe and Nothnagel, 1994), cell expansion (Zhu *et al*., 1993), and cell death (Schindler *et al*., 1995). All these effects might be mediated by signal transmission across the plasma membrane (Willats and Knox, 1996).

Recently, a glycoconjugate extracted from corms of the saffron plant (*Crocus sativus* L) has been isolated which is cytolytic to human tumour cells *in vitro*, and accounts for 0.5% (by weight) of the soluble fraction of corm extracts (Escribano *et al*., 1999*a*, 2000). Ultrafiltration analysis indicated it has an apparent molecular mass higher than 30 kDa. The polysaccharide moiety of the glycoconjugate accounts for 94.5% of the molecule, and its monosaccharide composition is dominated by Rha (36.4%) with Ara (18.8%), Fuc (15.2%), Xyl (11.0%), Gal (9.4%), Glc (4.4%), and uronic acids (4.8%) also present. The

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Abbreviations: AGP, arabinogalactan-protein; $(\beta\text{-D-Glc})_3$, $(\beta\text{-D-glucosyl})_3$ Yariv phenylglycoside; BSA, bovine serum albumin; FDA, fluorescein diacetate; $IC_{50}$ = concentration giving half-maximal inhibition; MS, Murashige and Skoog; Rha, rhamnose.

protein backbone accounts for the remaining 5.5% of the molecule, and is composed mainly of Asx, Ala, Glx, Gly, and Ser, which altogether make up 60% of the total residues. The composition of the protein resembles that of AGPs, but the carbohydrate composition does not match that of AGPs. The peptidic core of the proteoglycan appears to be composed of a 14 kDa polypeptide, the N-terminus of which is blocked (Escribano *et al.*, 1999*a*). Interestingly, one peptide obtained by endoproteinase Glu-C digestion of the reduced and *S*-pyridylethylated glycoconjugate, presents the amino acid sequence Ser–Ala–Pro–Ala, which has also been found in several peptides derived from AGPs (Du *et al.*, 1994, 1996*b*; Pogson and Davies, 1995; Gleeson *et al.*, 1989; Gester *et al.*, 1996). Callus cell cultures of corm also synthesize this compound, indicating that the glycoconjugate expression is maintained under *in vitro* conditions (Escribano *et al.*, 1999*b*).

In this paper, it is reported that this glycoconjugate causes the inhibition of root elongation and other morphological changes on *Nicotiana tabacum* and *Arabidopsis thaliana* seedlings. It also has toxic effects on tobacco cells and protoplasts.

## Materials and methods

### *Materials*

Corms of 'La Mancha' saffron (*Crocus sativus* L.) were acquired from farmers in Lezuza, Albacete (Spain), and stored at −80 °C until further use. MS media were purchased from ICN Biomedical Ltd. (Oxfordshire, UK), BSA, FDA, larch wood arabinogalactan, and gum arabic were obtained from Sigma (St Louis, MO, USA). All chemicals used were reagent grade or of highest quality available. Suspension-cultured cells of *N. tabacum* L. cv. 'Bright Yellow-2' tobacco cells (BY-2) were kindly supplied by Japan Tobacco Inc. (Tokyo, Japan) and used as the source of protoplasts for this study. Cells were cultured on modified MS medium (Murashige and Skoog, 1962) and subcultured every 7 d as described previously (Nagata *et al.*, 1981).

### *Purification of the saffron corm glycoconjugate*

The glycoconjugate was purified by size-exclusion and anion-exchange chromatography, followed by reversed-phase high-performance liquid chromatography. Chromatographic fractions were tested for cytotoxicity as described previously (Escribano *et al.*, 1999*a*). Briefly, saffron corms were homogenized in 50 mmol $m^{-3}$ TRIS-HCl buffer, pH 7.5, containing 1 mmol $m^{-3}$ phenylmethylsulphonyl fluoride and 5 mmol $m^{-3}$ 1,4-dithio-DL-threitol. The extract was centrifuged at 12 000 *g* and the supernatant was recovered and concentrated 50 times using 30 kDa molecular mass cut-off centrifuge filters. The fraction higher than 30 kDa was loaded on a Bio-Gel P-100 column and eluted with 100 mmol $m^{-3}$ ammonium acetate buffer, pH 6.6. Aliquots of all fractions were tested for cytotoxic activity on human cervical epithelioid carcinoma (HeLa) cells. The active fractions were applied on a Bio-Rad (Hercules, CA) Macro-Prep DEAE column, and the sample was stepwise eluted with each 0.4, 0.8 and 1.0 mol $m^{-3}$ NaCl in 0.02 mol $m^{-3}$ TRIS-HCl buffer, pH 8.0. A Hewlett Packard 1100 HPLC (Palo Alto, CA) connected on line with a photodiode array detector, and a Supelcosil LC-304 C-4 column were used for reversed-phase HPLC of the anion-exchange active fractions. The column was equilibrated with water and eluted in a acetonitrile gradient. Fractions were recovered and the elution of the molecules was monitored by measuring absorbance at 214 nm. A major homogeneous peak, which corresponded to the proteoglycan was obtained, indicating it is composed of a single molecular species (Escribano *et al.*, 1999*a*).

### *Analysis of the effect of the glycoconjugate on seedlings root growth*

Twenty-five seedlings of *N. tabacum* cv. 'Petite Havana' and 25 of *A. thaliana* cv. 'Columbia' were grown for up to 30 d in 15 $m^{-3}$ Falcon tubes on MS medium containing 7 g $m^{-3}$ agar, 16 h photoperiod (50 μmol $m^{-2}$ $s^{-1}$) at 22 °C. Glycoconjugate was added to molten media to final concentrations of 0.1, 1.0, 10, and 100 μg $m^{-3}$. The root length of seedlings was determined after 30 d. Gum arabic (100 μg $m^{-3}$) was used in control experiments. For microscopic examination, roots of *N. tabacum* seedlings were prepared by rinsing briefly in an aqueous solution of 0.1% (v/v) Triton X-100 to remove excess agar prior to mounting directly on slides with large cover slips. Roots were them viewed on the plates and photographed at different magnifications on an inverted microscope (Nikon Eclipse TE 300) equipped with phase-contrast condenser and Ph objectives, using Kodak Gold 400–6 film for photographs.

### *Viability and cytolysis assays on plant cells and protoplasts*

Protoplasts from BY-2 cell line were prepared essentially according to Murata *et al.* (Murata *et al.*, 1994). Five-day-old tobacco BY-2 cultured cells or protoplasts isolated from these cells were incubated for 1 h at 25 °C with different concentrations (250, 50, 10, 2, 0.05, 0.015, and 0 μg $m^{-3}$) of saffron glycoconjugate in BY-2 cell culture medium for cells, and in washing medium plus osmoticum for protoplasts. After incubation, the viability of cells and protoplasts was assessed by FDA staining (Larkin, 1976), and expressed as the percentage of viable cells of the total cell number analysed (as described by Serpe and Nothnagel, 1994). The total number of cells/protoplasts was estimated, as well as the survival rate for each treatment, using a haemocytometer. The experiments were repeated three times. Larch wood arabinogalactan was used in control experiments. Photographs were taken with a light microscope Olympus BH-2 equipped with a mercury vapour lamp and an Automatic Exposure Photomicrography unit PM-10AD. The concentration of protein released to the culture medium from tobacco cells and protoplasts exposed to different glycoconjugate concentrations (0.015–250 μg $m^{-3}$), for 1 h at 25 °C, was determined by the Bradford assay (Bradford, 1976), using a calibration curve constructed with different concentrations of BSA.

## Results

### *Effect of glycoconjugate treatment on root formation* in vitro

*N. tabacum* and *A. thaliana* seeds were germinated *in vitro* in the presence of glycoconjugate concentrations ranging from 0–100 μg $m^{-3}$. Seed germination was not affected by the treatment, with all roots emerging at the same time. During subsequent growth, the presence of glycoconjugate in the culture medium produced a significant

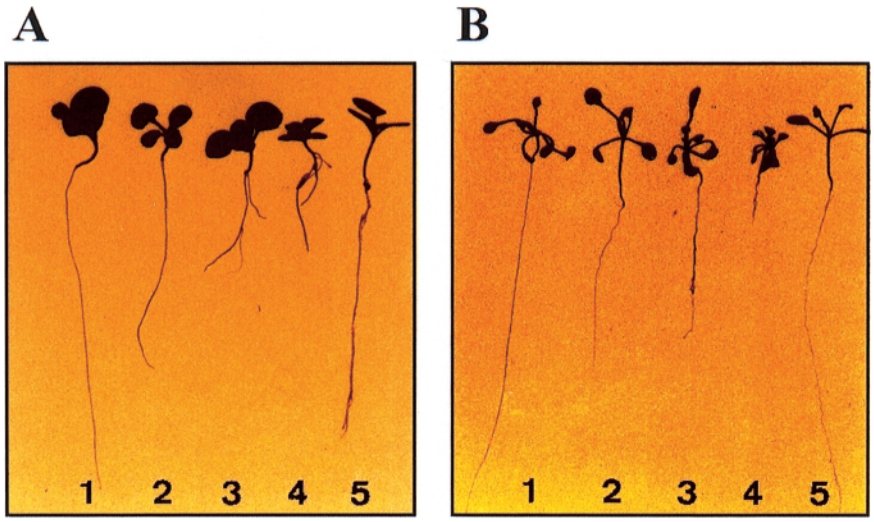


**Fig. 1.** Effect of saffron corm glycoconjugate on growth of roots of *N. tabacum* (A) and *A. thaliana* (B). Seedlings were grown *in vitro* without glycoconjugate (1) or in the presence of 1.0 μg $m^{-3}$ (2), 10 μg $m^{-3}$ (3) or 100 μg $m^{-3}$ of glycoconjugate (4) or 100 μg $m^{-3}$ of gum arabic (5), for 30 d at 25 °C.

concentration-dependent reduction of root elongation (Figs 1, 2). Roots of seedlings grown for 30 d in the presence of 100 μg $m^{-3}$ glycoconjugate were about one-third of the length of those of untreated seedlings. Growth of seedlings exposed to less than 0.1 μg $m^{-3}$ glycoconjugate did not affect root growth in a significant manner (data not shown). Control gum arabic at 100 μg $m^{-3}$ did not show any significant effect on root growth (Figs 1, 2).

Under phase-contrast microscopy, roots of seedlings exposed to 100 μg $m^{-3}$ gum arabic did not show any morphological alteration (Fig. 3A). However, seedlings treated with 0.1 μg $m^{-3}$ glycoconjugate showed epidermal cell bulging at the root hair zone (Fig. 3B). At concentrations higher than 10 μg $m^{-3}$ roots were devoid of hairs (Fig. 3C). Roots grown on medium containing glycoconjugate at a concentration of 100 μg $m^{-3}$ showed enlarged vascular tissues, probably due to cell wall thickening (Fig. 3C). Some epidermal cell areas of the extension and determination zones had deteriorated and degeneration of root cap cells was also observed (data not shown).

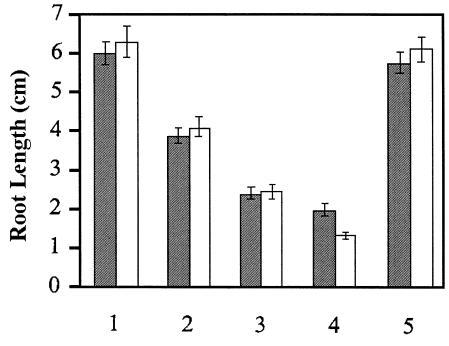


**Fig. 2.** Length of roots of seedlings of *N. tabacum* (shadowed bars) and *A. thaliana* (white bars) grown *in vitro* in the absence of glycoconjugate (1) or in the presence of 1.0 μg $m^{-3}$ (2), 10 μg $m^{-3}$ (3) or 100 μg $m^{-3}$ of glycoconjugate (4) or 100 μg $m^{-3}$ of gum arabic (5). Treatments were performed as indicated in Fig. 1. Error bars indicate the standard errors of the means ($n = 5$ for all treatments).

In order to test if the effects of glycoconjugate were reversible, some seedlings grown for 30 d on a medium containing glycoconjugate at a concentration of 100 μg $m^{-3}$ were transferred to glycoconjugate-free medium. After 2 weeks, rapid proliferation of multiple roots (between 2 and 3 cm long) was observed, indicating that the absence of glycoconjugate promotes the development of new roots (data not shown).

### In vitro *cytotoxic activity of the glycoconjugate on tobacco cells and protoplasts*

Treatment of tobacco cells or protoplasts with different concentrations of glycoconjugate produced a concentration-dependent decrease in cell viability (Fig. 4). The estimated $IC_{50}$ values were 0.5 and 2 μg $m^{-3}$ for cells and protoplasts, respectively. Larch wood arabinogalactan did not exert any effect on cell viability (Fig. 4). Microscopic examination of tobacco cells exposed for 1 h to a glycoconjugate concentration equal to its $IC_{50}$ revealed that about 50% were reduced in size, had condensed cytoplasm and loss of regular cell morphology (Fig. 5A). The use of FDA, a vital dye, showed that the glycoconjugate caused death of some of the cells (Fig. 5B). In addition, microscopy of tobacco protoplasts treated for 1 h with a glycoconjugate concentration equal to its $IC_{50}$, showed that about 50% of the protoplast population presented morphological changes (Fig. 5C). The use of FDA indicated that the glycoconjugate caused death of protoplasts (Fig. 5D). Treated protoplasts showed disorganization of the cytoplasm (Fig. 5E, F).

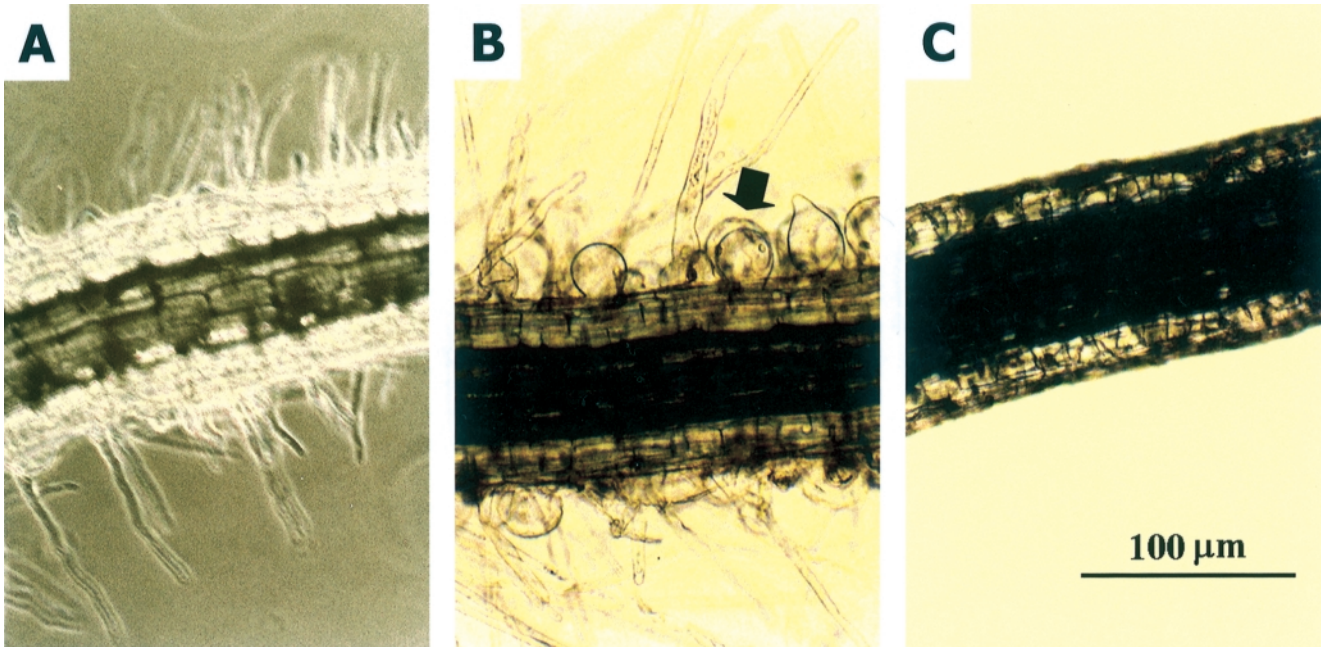


**Fig. 3.** Phase-contrast microphotographs of the primary maturation zone showing the effect of saffron glycoconjugate on root morphology of *N. tabacum* seedlings. (A) Roots of seedlings exposed for 30 d to 100 µg $m^{-3}$ gum arabic. (B) Roots exposed under the same conditions to 0.1 µg $m^{-3}$ glycoconjugate showed a remarkable epidermal cell bulging (arrow). (C) Hairs were absent and the central cylinder was thickened in roots treated with 100 µg $m^{-3}$ glycoconjugate for 30 d. Scale bar = 100 µm.

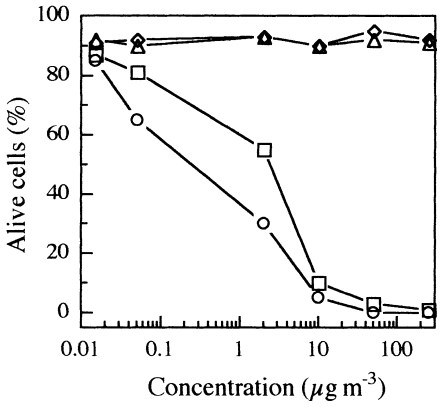


**Fig. 4.** Cytotoxic effect of the glycoconjugate on tobacco cells and protoplasts. 5-d-old tobacco BY-2 cultured cells (○) or protoplasts (□) from these cells were treated for 1 h at 25 °C with different glycoconjugate concentrations (0.015–250 µg $m^{-3}$), in BY-2 cell culture medium for cells, and in washing medium plus osmoticum for protoplasts. Cell and protoplast viability was assessed by fluorescein diacetate uptake (according to Larkin, 1976). As controls, tobacco cells (Δ) and protoplasts (◇) were treated with larch wood arabinogalactan. Experiments were performed in triplicate.

As it has been found that this compound is cytolytic to human cells *in vitro* (Escribano *et al.*, 2000), the authors investigated whether it exerts the same effect on plant cells. Tobacco cells and protoplasts exposed to the glycoconjugate showed a dose-dependent release of intracellular proteins to the culture medium, indicating that cell lysis was produced. Controls performed with BSA or larch wood arabinogalactan were negative (Fig. 6).

## Discussion

A report is given here of a reversible, dose-dependent reduction of root growth of *N. tabacum* and *A. thaliana* seedlings cultured in media containing different concentrations of a saffron corm glycoconjugate (0.1–100 µg $m^{-3}$) which chemically differs from typical AGPs (Escribano *et al.*, 1999*a*). At the microscopic level bulging of the root outer epidermal cells has been observed when tobacco or *A. thaliana* seedlings were grown in the presence of 0.1 µg $m^{-3}$ glycoconjugate. Identical morphological changes have been observed in roots of *A. thaliana* treated with Yariv phenylglycosides (β-D-Glc)$_3$, a kind of synthetic molecule that specifically interacts with AGPs by a mechanism that is not fully understood (Willats and Knox, 1996; Ding and Zhu, 1997). Perturbation of AGPs on the surface of cells by addition of (β-D-Glc)$_3$ reduces root growth (Willats and Knox, 1996; Ding and Zhu, 1997), inhibits cell division and cell elongation (Serpe and Nothnagel, 1994; Willats and Knox, 1996), and kills cells in culture (Langan and Nothnagel, 1997). Moreover, the morphological alterations produced by the addition of low concentrations of the saffron *Crocus* glycoconjugate or (β-D-Glc)$_3$ resemble the phenotype of the *reb* (root epidermal cell bulging) mutant of *Arabidopsis* (Baskin

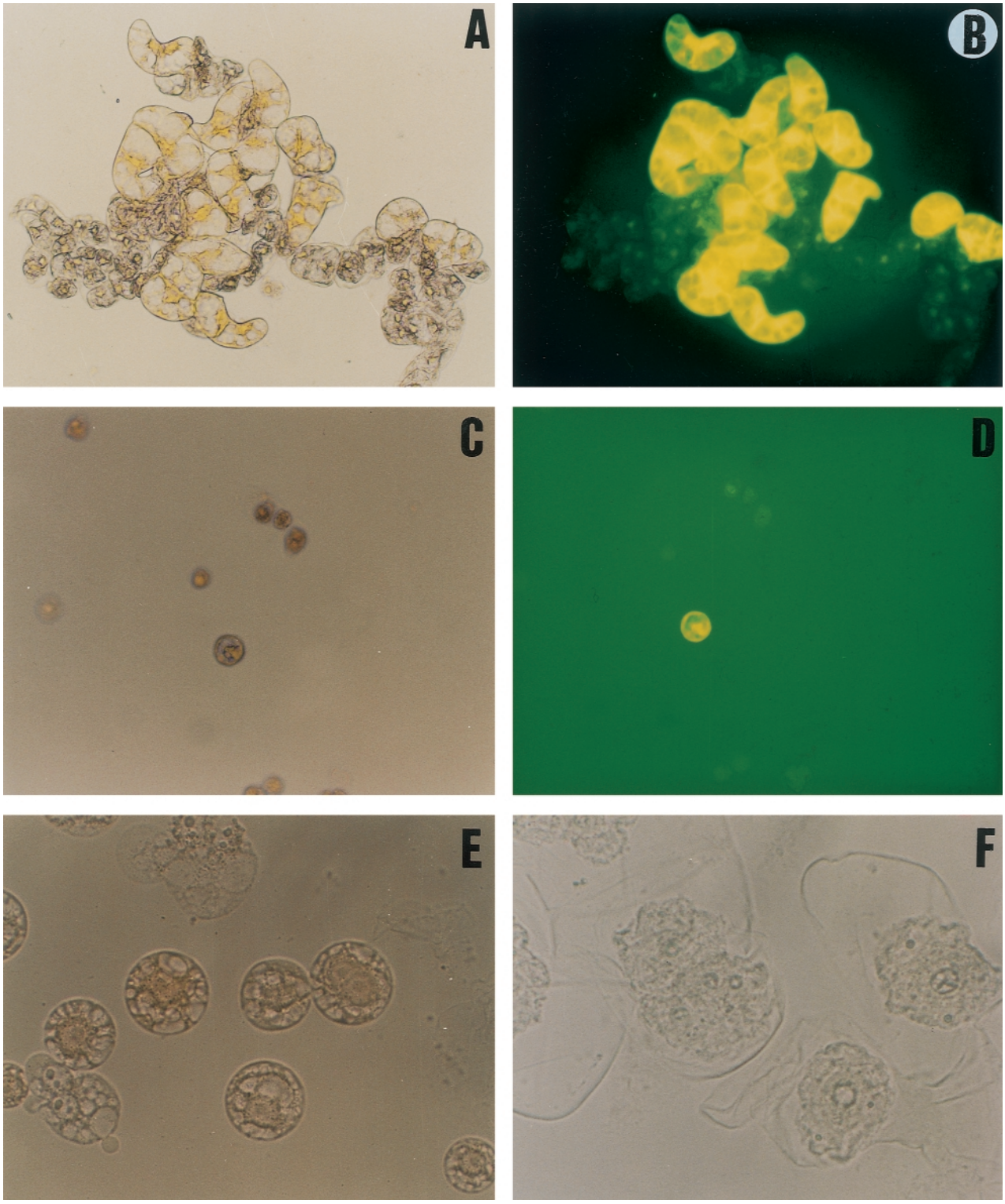


**Fig. 5.** Effect of glycoconjugate on cultured tobacco cells and protoplasts. 5-d-old tobacco BY-2 cultured cells or protoplasts from these cells were exposed for 1 h at 25 °C to the glycoconjugate in BY-2 cell culture medium for cells, and in washing medium plus osmoticum for protoplasts. Bright field (A) and fluorescence microscopy (B) of tobacco cells with 0.5 μg $m^{-3}$ glycoconjugate. Bright field (C) and fluorescence (D) microscopy of protoplasts treated with 2.0 μg $m^{-3}$ glycoconjugate. Bright field microscopy of control protoplasts (E) or protoplasts exposed to 2.0 μg $m^{-3}$ glycoconjugate (F). Magnification: (A), (B), (C) and (D) ×100; (E) ×200; (F) ×400.

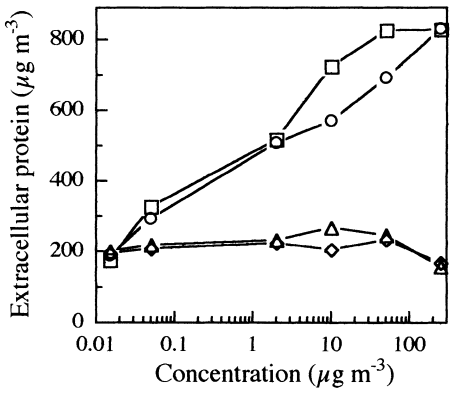


**Fig. 6.** Release of intracellular proteins to the culture medium after glycoconjugate treatment of tobacco cells and protoplasts. 5-d-old tobacco BY-2 cultured cells (□) or protoplasts from these cells (○) were treated for 1 h at 25 °C with different glycoconjugate concentrations (0.015–250 μg $m^{-3}$),in BY-2 cell culture medium for cells, and in washing medium plus osmoticum for protoplasts. After treatment cells were centrifuged and concentration of released proteins was determined in triplicate by the Bradford assay, using aliquots of the obtained supernatants. As control, tobacco cells (Δ) or protoplasts (◇) were treated with larch wood arabinogalactan.

*et al.*, 1992), which has been described as presenting defective or missing AGPs in roots (Ding and Zhu, 1997). According to these results one can speculate that the glycoconjugate from saffron corms at low concentrations might behave as an AGP-binding molecule, perturbing the normal interaction between root AGPs and other cell-surface components that link directly or indirectly to the plasma membrane, as probably (β-D-Glc)$_3$ does (Nothnagel, 1997). A more direct effect of the corm glycoconjugate on the cell surface could be considered as an alternative hypothesis. Epidermal cell bulging has also been reported in the apical meristem of tomato plants treated with expansin, an extracellular protein that increases plant cell wall extensibility *in vitro* (Fleming *et al.*, 1997). Despite the fact that involvement of AGPs in root formation has been largely highlighted, non-AGP or AGP-related molecules have been described as having a direct inhibitory effect on root growth. As far as is known, the compound isolated from the saffron corm is the first plant glycoconjugate with such effect to have been reported.

It was also detected that seedlings grown at concentrations of the glycoconjugate higher than 10 μg $m^{-3}$ were completely devoid of root hairs. At 100 μg $m^{-3}$ glycoconjugate, the cell walls of the root vascular tissues were thicker and, overall, the vascular tissue was enlarged. This finding could support the hypothesis suggesting that AGPs may behave as pectin-binding proteins, involved in the secondary thickening of xylem vessels and lignin deposition (Dolan and Roberts, 1995; Dolan *et al.*, 1995). Although the molecular mechanisms of this effect are still unknown, these results add more evidence to the frequently reported association between secondary thickening in root development and AGP presence (Nothnagel, 1997).

The effect of the saffron glycoconjugate on plant cell viability was evaluated using a tobacco cell line and its derived protoplasts, at concentrations ranging from 0.015–250 μg $m^{-3}$. These results show that plant cells are very sensitive to this compound. The lower susceptibility of tobacco cells to the glycoconjugate ($IC_{50}=0.5$ μg $m^{-3}$) compared to protoplasts ($IC_{50}=2$ μg $m^{-3}$), suggests that the cell wall does not protect the cell against glycoconjugate action. Again, cell death has also been observed in *Rosa* suspension cultures as a response to AGP perturbation, after exposure to (β-D-Glc)$_3$, but the mechanism by which these cells are killed has not been established (Langan and Nothnagel, 1997).

Measurements of released intracellular proteins showed that the saffron glycoconjugate induces cell lysis on tobacco cells and protoplasts. These results show that cells are directly damaged by plasma membrane alterations and cytoplasmic collapse, in a process of cell death or necrosis. In previous works it has been found that human tumour cells exposed to this compound show a remarkable swelling after a few minutes of incubation, resembling cell bulging (Escribano *et al.*, 1999*a*, 2000). Measurements of intracellular calcium fluctuations and release of lactate dehydrogenase revealed that this compound causes plasma membrane damage on tumour cells, allowing movements of both calcium and macromolecules which leads to cell lysis (Escribano *et al.*, 2000). Although structurally similar only in gross features, plant and animal macromolecules share localization and some functional characteristics. This has led to the suggestion that alterations in plasma membrane permeability could be implicated in the bulging of root epidermal cells at low glycoconjugate concentrations (0.1 μg $m^{-3}$), whereas cell death is produced at concentrations above 10 μg $m^{-3}$. This cytotoxicity could explain the absence of root hairs and the deterioration of some epidermal areas and root caps. Due to the size of the glycoconjugate (more than 30 kDa) it is believed to be unlikely that the compound could get through the walls of tobacco cells, but that it might interact with or bind particular molecules at the cell surface, as has been hypothesized for AGPs (Nothnagel, 1997).

At this time, it is not known whether the intense cytotoxic action of the glycoconjugate is related to its biological function or, on the contrary, it is a consequence of *in vitro* conditions. On isolated tobacco cells and protoplasts the glycoconjugate works as a cytotoxin, whereas on root tissues the compound mimics the effect of (β-D-Glc)$_3$, a molecule that binds most AGPs. Further research is required to understand these points completely.

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