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Secretion of Specific Cytotoxic Products by Plant Storage Organs after Wound-Desiccation Stress

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ABSTRACT

After a wound-desiccation stress, plant tissues can secrete products that have specific cytotoxicity against melanoma B16 cells. Healthy plant or animal cells were not susceptible to these products. Secretion of the active product varied with the plant species and the plant organs. The secretion of tubers of yam (*Dioscorea cayenensis* Lam), cassava (*Manihot esculenta*), Jerusalem artichoke (*Helianthus tuberosus*), ginger (*Zingiber officinale*) and potato (*Solanum tuberosum* L.) showed activity against murine melanoma B16 cells, but not against immortalized fibroblast L929 cells. A decrease of 20 to 30% of the B16 clonogenicity was observed, while the treated L929 fibroblast cells did not show any significant difference with the control cell colonies. In contrast, the secreted products of roots of beet (*Beta vulgaris*) and tubers of sweet potato (*Ipomoea batatas*) only showed slight cytotoxicity. Secretion of the active product was associated with the storage tissues. The physiology of the plant, such as the light growth conditions (for hypocotyls of sunflower, *Helianthus annuus* L., and tomato, *Lycopersicon esculentum* Mill), or dormancy (for Jerusalem artichoke tubers, *Helianthus tuberosus* L.), was involved in the secretion process of an active product. The active agent was associated with a protein complex. The secretion of the cytotoxic products against melanoma B16 cells, thus, can be performed through a specific stress on the storage organs of plants usually cultivated.

Keywords: abiotic stress, plant secretion, tumour cell
Abbreviations: MEM, minimum Eagle's essential medium; **PAGE**, polyacrylamide gel electrophoresis; **SDS**, sodium dodecyl sulfate

INTRODUCTION

A major problem with present day cancer chemotherapy is the serious deficiency of active drugs for curative therapy. Natural products have led to the discovery of the majority of anticancer agents presently in use and account for a significant fraction under development (Cragg *et al.* 1997). Plants are a valuable and assessable source of new natural products. There is increasing interest in the potential of plant medicines to be used in chemotherapy and this has led to investigations of a range of plant extracts and other products that have been used traditionally (Valeriote *et al*. 2002; Lusby *et al*. 2006). The potential medicinal value and wide acceptability of plants have attracted interest in the search for biologically active substances from them. Extracts from a broad spectrum of plant species contain substances that possess antitumor activity. Most of the active compounds in these extracts still remain unidentified and their presence is detected by biological tests only. The structure and the mechanism of action of others have been elucidated and some of them are currently used as drugs in chemotherapy (Dzhambazov *et al*. 2002).

In contrast, when plants are stressed they can synthesize and secrete a large variety of products, which is partly correlated to the type of stimulus. Most of the secreted products are then related to a plant defence process. They involve different kinds of molecular species. Previously, we had shown that the wounding and desiccation of plant tubers allowed the secretion of cytokinin-like products that show a cytotoxic feature, which is correlated to the molecular structure (Griffaut *et al*. 2004), but also the secretion of enzymatic proteins such as new types of protease inhibitors (Ledoigt *et al*. 2006) and a superoxide dismutase (Griffaut *et al*. 2007).

Here, we show that the stress of plant tissues can trigger the secretion of specific cytotoxic products against melanoma cells. A type of each main plant tissue was tested and different plant species were used in order to characterize the best production of the cytotoxic agents.

MATERIALS AND METHODS

Plant materials

Tubers, roots, leaves and seedlings were obtained from several commercial plant species from different families, as shown in **Table 1**.

Jerusalem artichoke tubers (*Helianthus tuberosus* L.) were grown in fields, harvested at the autumn and stored at 4°C (duration: 3 months) for breaking the dormancy. Seeds of sunflower (*Helianthus annuus* L.) and tomato (*Lycopersicon esculentum* Mill) were washed in sodium hypochloride (6%) for 30 min, and then rinsed for 15 min in sterile distilled water, three times.

The seeds were put on a latticework in a Knop medium, for germination in a growth chamber. The akenes of sunflower were allowed to germinate for 24 h in sterile water prior to transfer to Knop's medium (Bourgin and Nitsch 1967; Nitsch and Nitsch 1969). Hypocotyls of sunflower (*Helianthus annuus* L.) and tomato (*Lycopersicon esculentum* Mill) were subsequently grown either in a light or in a dark room, at 25°C.

Plant secretion products

Plant roots and tubers were washed by water then put into a sodium hypochloride (6%) solution for 30 min. They were then washed in sterile distilled water for 15 min, three times.

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Table 1 Plant materials and plant secretion product activity.

Common name	Latin name	Family	Organ used	Stress period	IC_{50} B16 cells	% B16 * clonogenicity	% L 929* clonogenicity
Monocotyledons							
Yam	*Dioscorea cayenensis* Lam	Dioscoreaceae	Tuber				
Ginger	*Zingiber officinale*	Zingiberaceae	Tuber	2h	195 +/- 9µl	47 +/- 5	100 +/- 5
Dicotyledons							
Beet	*Beta vulgaris* L.	Chenopodiaceae	Root				
Cassava	*Manihot esculenta*	Euphorbiaceae	Tuber				
Sweet potato	*Ipomoea batatas*	Convolvulaceae	Tuber				
Potato	*Solanum tuberosum* L.	Solanaceae	Tuber	2h	70 +/- 9µl	28 +/- 11	100 +/- 5
Tomato	*Lycopersicon esculentum* Mill	Solanaceae	Hypocotyl				
Jerusalem artichoke	*Helianthus tuberosus* L.	Asteraceae	Tuber	2h	144 +/- 9µl	51 +/- 4	84 +/- 5
Chicory	*Cichorium intybus*	Asteraceae	Root	2h	171 +/- 9µl	41 +/- 5	98 +/- 4
Sunflower	*Helianthus annuus* L.	Asteraceae	Hypocotyl				

*: melanoma B16 cells and fibroblast L929 cells

The parenchyma of the tubers and roots were cut in parallelepipeds (2 cm × 1 cm × 1 cm). Whole hypocotyls (about 2 cm) were used when specified.

The plant fragments were washed by sterile water and placed for 24 h in a sterile culture tube (3 cm × 15 cm), for air desiccation. Earlier, the desiccation step was performed in order to get a scar tissue preventing cellular leakage. But, the previous studies showed that a desiccation step was the first part of stress process needed to obtain secreted products; the second important part was the hydration step (Ledoigt *et al*. 2006; Griffaut *et al*. 2007).

Each explant was transferred into a tube containing 3 ml of Knop's medium (½) at pH 5.5 for 2 or 24 h at 24°C, as specified in the text. The secretion process was optimized by having a large living plant fragment in a small amount of medium (Ledoigt *et al*. 2006; Griffaut *et al*. 2007). Thus, about one third of the plant fragments were immersed. After 24 h, the plant pieces were removed and each solution was collected, filtered, centrifuged at 10,000 × *g* using a Sigma 2K15, rotor 12145 for 15 min, then dialysed to removed medium ions, for 4 h against a Tris-HCl buffer (2 mM, pH 7.4) with constant stirring, with renewed medium, at 4°C (membrane of dialysis Spectra / Por MWCO: 6 - 8,000).

The dialysed products were filtered on a cartridge Millipore (0.22 µm) for sterilisation, then stored in a refrigerator till use.

The dialyzed exudates were then centrifuged on a filter device (AMICON Ultra-15 10.000 MWCO, Millipore) allowing the protein fraction to concentrate in the upper part of the ultrafiltration cartridge, and to remove the small molecules (less than 10 kDa). The products, 15 ml per tube, then were centrifuged for 10 min at 2,800 × *g* (Multifuge 3S-R Heraeus, rotor Sorvall Heraeus 6445). The final volume of each fraction was 2 ml, and was sterilized through a membrane (syringe Millex HV Filter unit 0.45 µm or cartridge Millipore 0.22 µm).

Aliquots of the dialysed solutions were done. Some aliquots were treated by ammonium sulphate (60%). The salt solution was slowly added to the plant extracts, with gentle stirring. The mixtures were incubated for 1 h at 4°C, and then centrifuged (15 min, 10, 000 × *g*, 4°C). The supernatants were removed and the pellets were mixed with a Tris-HCl buffer (2 mM, pH 7.4). Both supernatants and pellets were dialysed for 24 h in a Tris-HCl buffer (2 mM, pH 7.4).

All other plant species were treated in the same way. Tuber parenchyma pieces of yam (*Dioscorea cayenensis* Lam), cassava (*Manihot esculenta*), Jerusalem artichoke (*Helianthus tuberosus*), ginger (*Zingiber officinale*), potato (*Solanum tuberosum* L. cv 'Agata'), roots of chicory (*Cichorium intybus*) and hypocotyls of sunflower (*Helianthus annuus* L.) and tomato (*Lycopersicon esculentum* Mill) were cut off for *in vitro* culture. Each piece of organ parenchyma (2 cm × 1 cm × 1 cm) was put into a tube containing sterile Knop's medium. Stress treatment (desiccation and moistening) and secreted products were obtained as described for *H. tuberosus* tubers.

Cell lines and culture medium

The murine melanoma B16 cells were a kind donation from Dr. Kirsten Falk and Dr. Olaf Rötzschke Max Delbrück Centre for Molecular Medicine Berlin-Buch.

The cells were cultured in MEM solution (Minimum Eagle's essential Medium, GIBCO), supplemented with 1% vitamins, 1% glutamine, 1% non-essential amino-acids, 10% Fœtal Veal Serum and 0.05% gentamycin (Calbiochem), in a humidified atmosphere containing 5% carbon dioxide, at 37°C.

The cells were grown in the whole MEM mixture, supplemented with 10% DMSO (dimethyl sulfoxide, Calbiochem), and stored in liquid nitrogen. After a quick defrost at 37°C, cells were put in a whole MEM solution. 24 h later, the medium was removed and a fresh MEM was added. When the cells were altogether joining, they were washed in a PBS solution (Phosphate Buffer Saline). The cells then were removed from the plates using a trypsin-EDTA (ethylene diamine tetraacetic acid) 5X mixture. Adding MEM allowed stopping the reaction. Cells were counted on a Malassez plate.

Clonogenicity

Two animal cell lines were used for this study, the murine melanoma B16 cells and the L929 fibroblast cell line. For each experiment, 150 cells were put into 2 ml MEM solution, in Petri dishes (40 mm in diameter). The dishes were placed in moist air supplemented with 5% $C0_2$, at 37°C, for 20 h, until the cells can be fixed on the plates. The plant extracts then were added into the medium at different amounts (100 and 200 µl). 10 days later, the plates were rinsed with PBS (Phosphate Buffer Saline), and cells were fixed with methanol, and stained with a 0.2% violet crystal solution. The cell generation was about 15 h. Only the colonies having more than 50 cells were taken in account for the clonogenicity. The standard errors were obtained as the result of at least three different treatments with the same excreta.

In vitro assay to test antitumour effect on plants

The crown-gall, a well-known model of plant tumours, was induced by *Agrobacterium tumefaciens* (strain B6 806) infection on pieces of Jerusalem artichoke tubers (*H. tuberosus* L) cultured in Knop's medium under controlled conditions (25°C) as previously described (Griffaut *et al.* 1994). Jerusalem artichoke was chosen for its high susceptibility to infection by *A. tumefaciens.* Tumour tissue can be obtained 10 to 15 days following inoculation. Cefotaxime (0.5 gl^{-1}) was then added to the Knop's medium to eliminate the agrobacteria. The tumour tissue grew on the parenchyma, which was maintained outside the Knop's mixture, and the non-transformed part of the parenchyma was thus immersed in the medium.

The cytotoxic activities of the dialyzed secretion solutions were tested on crown-gall tumour fragments, by direct application to the tumour tissue. The necrotic tissues were observed after 2 days.

Proteins analysis by one or two dimensions polyacrylamide gel electrophoresis (PAGEs)

In non-denaturing conditions, PAGEs were performed according to Clarke and Critchley (1992). The protein samples were separated on 10% polyacrylamide gel (4% stacking in Tris-glycine buf-

fer pH 6.8, and 10% separation gel) in Tris-glycine buffer (pH 8.3).

The SDS PAGEs (in Apelex model V10-CDC) were performed according to Laemmli (1970) modified by Schagger and Von Jagow (1987). The polypeptide samples were mixed up with an equal volume of a solubilisation solution (5% v/v 2-mercaptoethanol, 10% w/v glycerol, 4% SDS and 0.01% w/v bromophenol blue, in 0.5M Tris-HCl buffer pH 6.8) and samples were heated in boiling water bath for 2 min. An equal volume (5 µl) for each sample was separated on 12.5% polyacrylamide gel (4% stacking gel in a SDS-Tris-glycine buffer pH 6.8 and 12.5% separation gel, in a SDS-Tris-glycine buffer pH 8.8).

The gels were stained either with silver nitrate, according to Blum *et al* (1987) or with Coomassie Blue R250 (BioRad) (Irie *et al.* 1982).

Statistical analyses

Values were expressed as the mean of three measurements for each treatment. Colonies that contained more than 50 cells were counted and cell clonogenicity (percentage of cell forming colonies relative to untreated control) was calculated. Each point is the average of at least three independent repeat (+/- SD).

RESULTS

Plant secretion of the cytotoxic product

The products that were secreted after a wounding-desiccation stress on different organs from several plant species, have been used to study the clonogenicity of two cell strains, murine melanoma B16 cells and immortalized fibroblast L929 cells (**Table 1**). The secretion products of yam tubers (*Dioscorea cayenensis* Lam), cassava tubers (*Manihot esculenta*), beet (*Beta vulgaris*) and tubers of sweet potato (*Ipomoea batatas*) have shown toxicity against murine melanoma B16 cells, but did not display any toxicity against immortalized fibroblast L929 cells (**Fig. 1**). A similar result was obtained with excreted products of tubers of Jerusalem artichoke (*Helianthus tuberosus*) (**Fig. 2**), ginger (*Zingiber officinale*) (**Fig. 3**) and potato (*Solanum tuberosum* L.) (**Fig. 4**). A decrease of 20 to 30% of the B16 clonogenicity was then observed, when the treated L929 fibroblast cells did not show any significant difference with the control cell colonies. The concentration of the active product in the secretions was therefore dependent of the plant species and displayed, for instance, a higher cytotoxicity with potato tubers than with other studied plant tubers (**Table 1**).

Ginger (*Z. officinale*) tuber secretions had shown a high cytotoxicity on melanoma B16 cells (20% mortality) with no significant effect on fibroblasts for a treatment with 100 µl secretion products (**Fig. 3**). A greater amount of product, 200 µl, increased the inhibition of B16 cell colony forming, but became cytotoxic for L929 cells (**Fig. 3**).

The active product can be secreted after 2 h hydration. In **Table 1**, activities of secreted products of the best plant producers were given after a 2 h hydration of stressed parenchyma. A slight increase of production was observed until 24 h, but the secreted active agent became less active after 48 h.

Physiological features of secreted cytotoxic products

Jerusalem artichoke tubers (*H. tuberosus*) products have shown a great effect on melanoma cells (90% mortality) with no significant action on fibroblasts treated with 100 µl secreted product (**Fig. 2**). This activity was dependent of the physiological state of tubers; when dormancy has been broken by a cold period (4°C) for 3 months, non-dormant tubers were shown to secrete a more cytotoxic product than dormant ones (data not shown). To investigate the antitumor effect of secreted products on plants, experiments were performed using crown-gall tissue obtained from *H. tuberosus* tubers previously infected by *Agrobacterium tumefaciens*. Product secreted by the parenchyma of the Jerusalem arti-

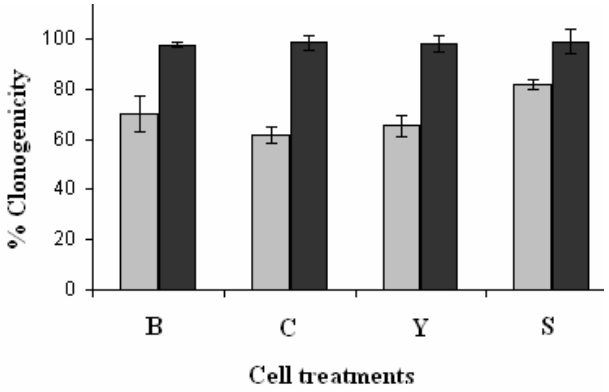

Fig. 1 Colony-forming study with secreted product from different plants. Products were secreted after a wounding-drying stress and 24 h hydration. Secretion of roots of beets (*Beta* vulgaris) (B) and tubers of yam (*Dioscorea cayenensis* Lam) (Y), cassava (*Manihot esculenta*) (C) and sweet potato (*Ipomoea batatas*) (S), were dialyzed and used against melanoma B16 cells (grey bars) and fibroblast L929 cells (black bars).

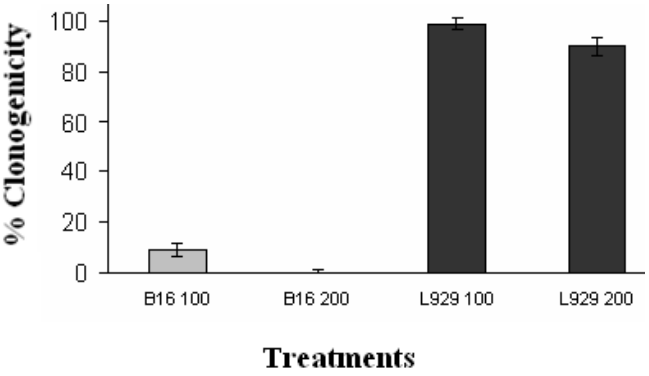

Fig. 2 Clonogenicity of B16 cells (grey bars) and L929 cells (dark bars) with products secreted by *H. tuberosus* non-dormant tubers. Products were secreted after a wounding-drying stress and 24 h hydration, and used after dialysis. The treatments were done using either 100 or 200 µl of the same secreted fraction.

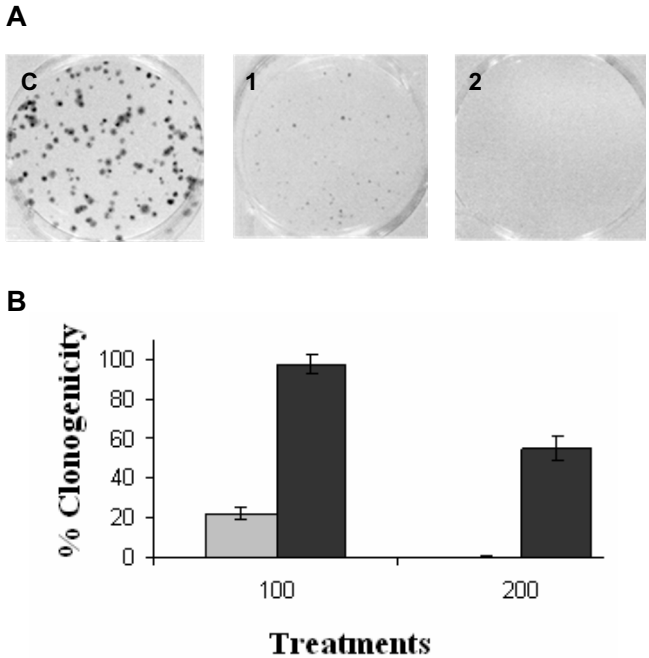

Fig. 3 Cytotoxicity of ginger (*Zingiber officinale*) products. Clonogenicity of B16 melanoma cells was observed after treatment by two different amounts of secreted proteins: 100 µl (**A**: plate 1; B: 100) and 200 µl (**A**: plate 2; B: 200) of purified products were mixed up with B16 cells (**B**, grey bars) and with L929 cells (L, black bars). Cell ratios were obtained with untreated B16 (**A**: plate c) and L929 cells respectively. Secretion products were obtained after a wounding-drying stress and a 24 h- hydration, and used after dialysis and Amicon concentration.

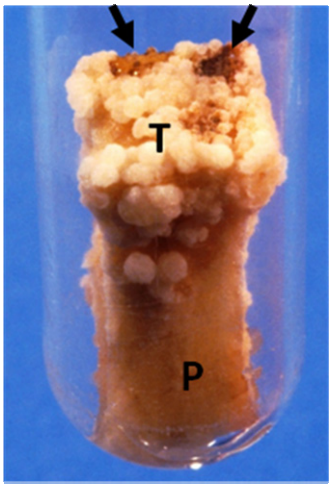

Fig. 4 Effect of secreted product of Jerusalem artichoke tubers (*H. tuberosus*) on crown-gall tissue. Plant tumours were induced by *Agrobacterium tumefaciens* strain B6 806 on pieces of tuber parenchyma (P) from Jerusalem artichoke (*Helianthus tuberosus* L), in a controlled growth chamber (25°C). Tumour tissue (T) was generated on the liquid-free part of tuber parenchyma (P); non tumour part of parenchyma was only immersed into medium. Two drops of 100 µl of purified active product were put on tumour cells and necroses were observed 2 days later (arrows).

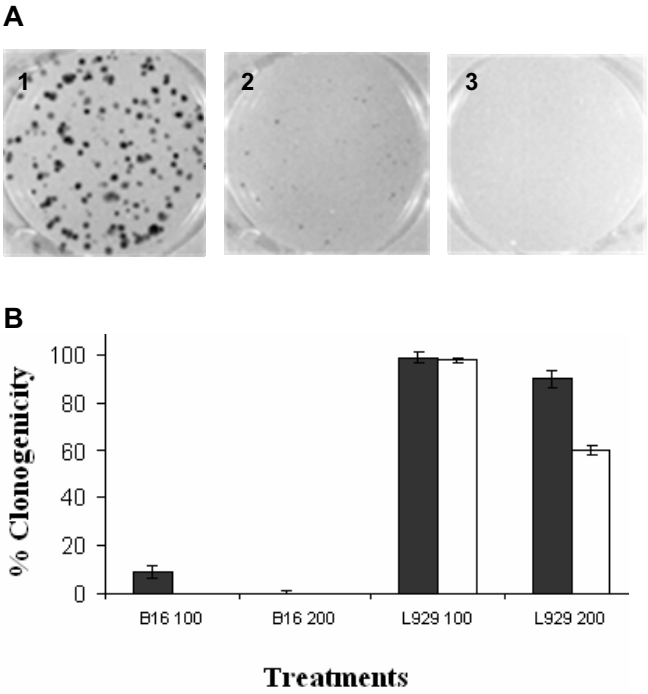

Fig. 5 Clonogenicity of B16 cells and L929 cells with a potato (*Solanum tuberosum* L. cv. 'Agata') product of unwashed cut parenchyma and a secreted product of washed potato parenchyma. (**A**) Colony-forming of melanoma B16 cells treated with a secreted product of unwashed parenchyma (plate 1) and with secreted product of washed parenchyma (plate 2). (**B**) Clonogenicity was measured on melanoma B16 cells or L929 cells treated with a secreted product of unwashed parenchyma (white bars) and with secreted product of washed parenchyma (black bars). The products were obtained after dialysis and concentrated by centrifugation in Amicon tubes. Clonogenicity of B16 and L929 control cells were given as 100%, respectively.

choke tubers was put on plant tumour tissues (**Fig. 4**). Necrotic tissue was observed at the site of the drops, two days after treatment (**Fig. 4**, arrows). Potato (*S. tuberosum*) tuber secretions displayed an inhibition of B16 cell clonogenicity, that was increased when the amount of product was doubled; but a slight cytotoxic effect was observed on L929 cell fibroblasts (**Fig. 5**). When fresh cut tubers were not washed prior to the desiccation stress, an increased cytotoxic effect

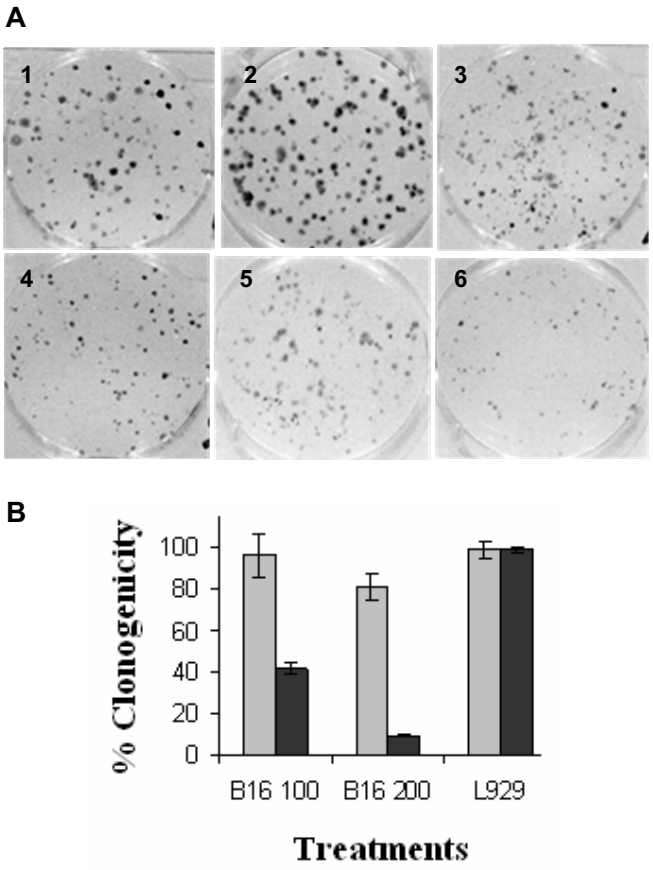

Fig. 6 Clonogenicity of murine melanoma B16 cells (B16) and fibroblast L929 cells (L929) with sunflower hypocotyls (*Helianthus annuus* L.) secretion products. (**A**) Colony forming of melanoma B16 tumour cells (control plates: plates 1 and 2; treated plates: plates 3, 4, 5 and 6). (**B**) Clonogenicity was calculated as a percent of the control plates. Two amounts of the products were used, 100 µl (**A**: Plates 3 and 4; **B**: B16 100) and 200 µl (**A**: plates 5 and 6; **B**: B16 200). The products were secreted after a wounding-drying stress and a 24 h hydration, and used after their dialysis and concentration. The clonogenicity was observed with the products of light-grown sunflower hypocotyls (**A**: plates 3 and 5; **B**: grey bars) or with those of dark-grown sunflower hypocotyls (**A**: plates 4 and 6; **B**: dark bars).

was observed in both cell colony forming and for both concentrations. Therefore a toxic product from broken cells was removed by washing the fresh tuber pieces (**Fig. 5**).

Effect of light on the production of cytotoxic agents

Hypocotyls, a botanical term for a part of a germinating seedling of a seed plant, of sunflower (*Helianthus annuus* L.) were light-grown then stressed; a treatment by secreted products had just produced a slight decrease of B16 cell colony forming when the amount of plant agent was increased (**Fig. 6**); in turn, such a treatment did not modify the treatment effect on L929 cell clonogenicity. In contrast, secretion products from stressed sunflower hypocotyls grown in a dark room have shown a greater cytotoxicity on tumour cells that was increased with a higher amount of product, without any toxicity on fibroblast colony forming (**Fig. 6**). The production of active secreted agent thus was shown to depend of the physiological state of the plant, especially with the light environment.

The secreted product of tomato (*Lycopersicon esculentum* Mill) hypocotyls has shown low or no toxicity against tumour cells (**Fig. 7**). However, light or dark conditions of plant cultures did not modify the cytotoxicity of secreted products.

Secretion product of chicory (*Cichorium intybus*) root had shown a high efficiency against tumour cells without a

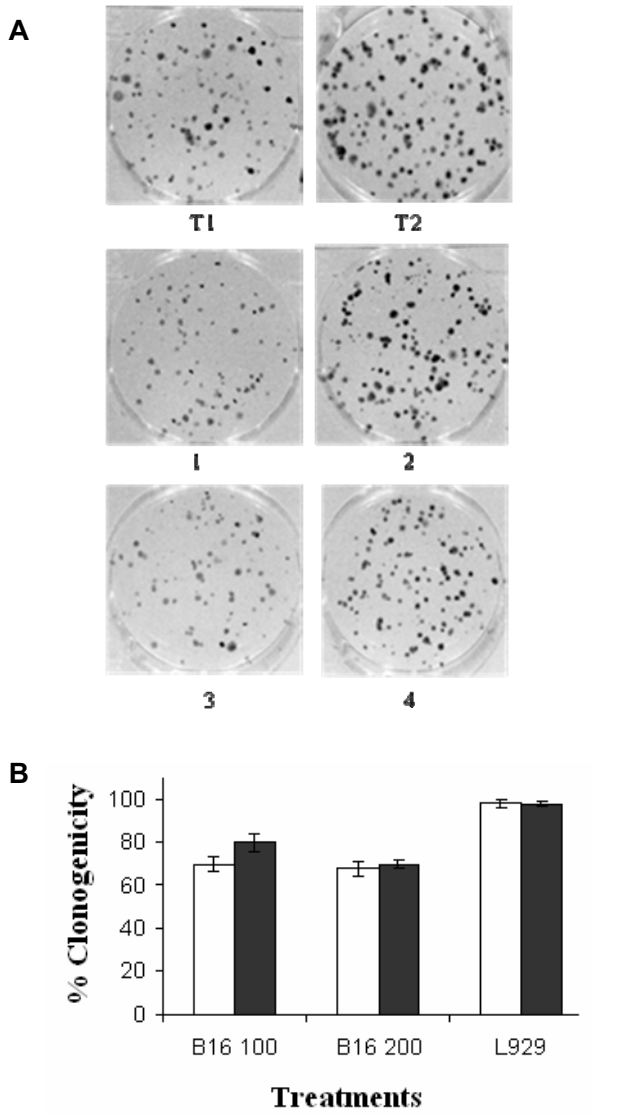

Fig. 7 Clonogenicity of B16 cells and L929 cells with the products secreted tomato hypocotyls (*Lycopersicon esculentum* Mill) following a light treatment. (**A**) Colony-forming of B16 melanoma cells. Plates 1 and 3 were treated with 100 and 200 µl, respectively, of dark-grown plant secretion product (T1 was the control plate). Plates 2 and 4 were treated with 100 and 200 µl, respectively, of light-grown plant secretion product (T2 was the control plate). (**B**) Clonogenicity was measured after treatment with the products from dark-grown (dark bars) and light-grown (white bars) tomato hypocotyls. Two amounts of product were used, 100 µl (100) and 200 µl (200). Products were secreted after a wounding-drying stress followed by a 24 h hydration, and used after their dialysis and concentration.

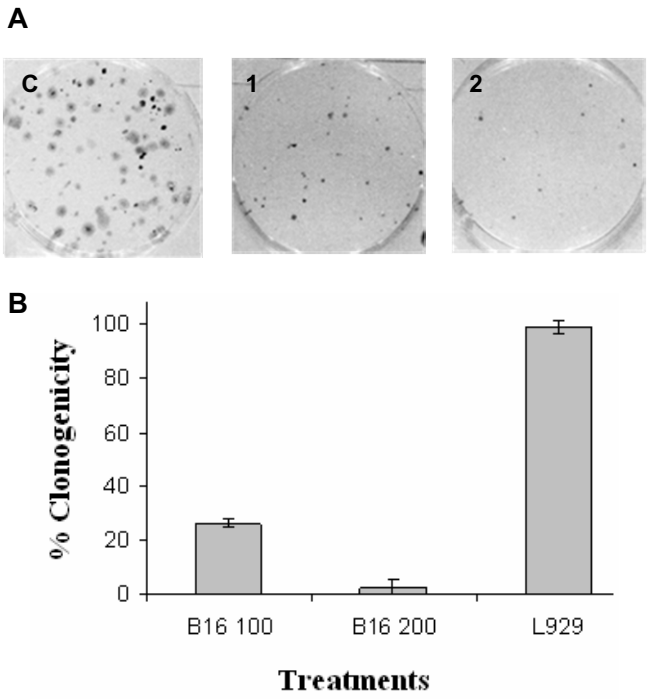

Fig. 8 Clonogenicity of B16 cells and L929 cells with products secreted by chicory roots (*Cichorium intybus*). (**A**) Colony-forming of B16 melanoma cells (C is the control untreated plate). Two amounts of the agent were used, 100 µl (**A**: plate 1; B: 100) and 200 µl (**A**: plate 2; **B**: 200). Products were secreted after a wounding-drying stress and 24 h hydration, and then used after dialysis and concentration.

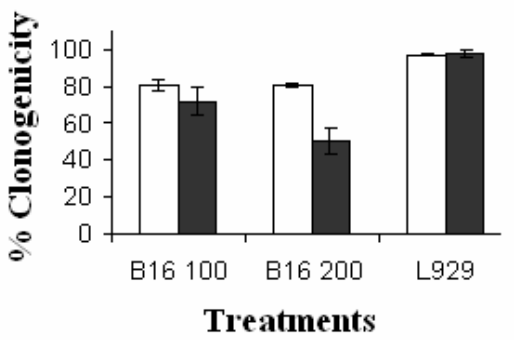

Fig. 9 Clonogenicity of B16 cells and L929 cells with the products secreted by the chicory leaves (*Cichorium intybus*). Secreted products from chicory leaves either of dark-grown (white bars) or of light-grown plants (dark bars) were used. Two amounts of the products were applied, 100 µl (100) and 200 µl (200). Products were secreted after a wounding-drying stress and 24 h hydration, and used after dialysis and concentration.

significant cytotoxicity against fibroblast cells (**Fig. 8**). In contrast, etiolated leaves of chicory had secreted a product that did not show any significant activity against tumour cells (**Fig. 9**). In turn, secretion product of green leaves of light-grown chicory had shown a slighter cytotoxicity against melanoma B16 cells than dark-grown leaf secreted product.

Composition of active products

Secreted products of potato (*S. tuberosum*) and ginger (*Z. officinale*) tubers have been treated with a solution of ammonium sulphate (60%) and, after dialysis, pellets and supernatants were used on cell cultures (**Fig. 10**). Supernatants did not show any significant cytotoxicity; the specific active products against tumour cells remained in the pellets. Active products could thus belong to a protein complex, for potato (**Fig. 10A**, **10C**) as for ginger (**Fig. 10E**) products. A second cycle of wounding-desiccation stress on potato tuber did not change the cytotoxicity features of secretion (**Fig. 10C**).

DISCUSSION

Plant stress and production

Different organs and tissues of several plant species were stressed and tested in order to analyse products of secretion. Different features of active product productions were then observed. The production of the cytotoxic agent was slight with some plants such as Beet roots (*B. vulgaris*), sunflower cotyledons (*H. annuus*), tomato leaves and hypocotyls (*L. esculentum*), tubers of cassava (*M. esculenta*), yam (*D. cayenensis*) and sweet potato (*I. batatas*). In turn, tubers of potato (*S. tuberosum*), ginger (*Z. officinale*) and Jerusalem artichoke (*Helianthus tuberosus*) had given a greater secretion of active agent.

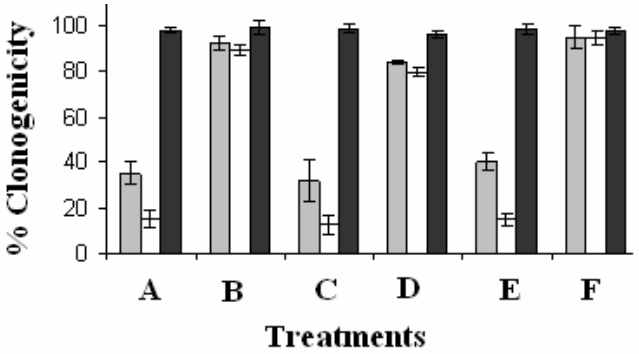

Fig. 10 Colony-forming study with different parts of the products secreted by plants, potato (*Solanum tuberosum* L. cv. 'Agata') (fractions A, B, C and D) and ginger (*Zingiber officinale*) (fractions E and F). The treatment was done after protein precipitation with ammonium sulphate, using either the pellet (fractions A, C and E) or the supernatant (fractions B, D and F) fractions. The study was performed on L929 fibroblast cells (dark bars) or on murine melanoma B16 cells (white and grey bars) by using two amounts of secretion fractions, 100 µl (grey bars), 200 µl (white bars). Potato extracts were obtained either after a double stress (A and B) or after a second double stress (C and D).

A cytotoxic activity was therefore observed either in secretion products of monocotyledonous or dicotyledonous plants. Production was mainly observed with plants that belong to more recent families, ends of phylum, such as Asteraceae, Solanaceae and Zingiberaceae. This could correspond to a more acute plant defence against an internal disease.

Characterization of secreted cytotoxic agents

Active products were related to protein complexes. Activity of products was shown to be dose-dependent against murine melanoma cells.

Stressed plants are able to synthesize and to secrete a large variety of molecules, such as cytokinin-like products (Griffaut *et al*. 2004) and proteins (Ledoigt *et al*. 2006; Griffaut *et al.* 2007).

Dietary intake of many fruits and vegetables has been shown to be associated with reduced risk of cancer (Singh *et al*. 2004). The activity against tumour of extracts of plant was extensively studied, and was correlated to different kind of molecules, such as phenols and flavonoids (Agarwal 2000; Annabi *et al*. 2002; Kanadaswami *et al*. 2005), terpenes (Kumar *et al*. 2004) and polysaccharides (Shin *et al*. 2004).

We showed that most of usual plants were able to secrete a cytotoxic product after a specific stress. Heat treatment or ammonium sulphate precipitation of concentrated secreted products from potato (*S. tuberosum*) and Jerusalem artichoke (*H. tuberosus*) allowed correlating cytotoxicity of secreted product to a protein complex. Polyacrylamide gel electrophoresis analyses of secreted products had shown main polypeptides in each sample that showed a different molecular weight according to plant species. Some polypeptides have been partly sequenced and displayed different type of proteins (**Table 2**).

An increase of product amount was sometimes shown to be slightly cytotoxic against fibroblast L929 cells. This could be due to the presence of small amount of non-specific cytotoxic contamination that remains after dialysis and purification of active product. For instance, when broken cell content was not removed from potato tuber fragments by washing the fresh cut, a non-specific cytotoxic effect of secretion product was observed (**Fig. 5**). Therefore, the use of active product needs a great purification for a better efficiency.

Cytotoxicity and plant physiology

A double stress, wounding and desiccation, of plant tissue allowed the secretion of products that are specifically active against animal (murine melanoma B16 cells) and plant (crown-gall) tumour cells, but are not toxic against non-transformed cells, L929 fibroblast cells and plant parenchyma tissues.

Activities against melanoma B16 cells were lower after a 2 h hydration treatment (**Table 1**) than after 24 h (**Figs. 2** and **3**). But the shortest period of induction allowed gaining times for the production of the active agent.

Storage organs, such as tubers and roots, were shown to be the best producers, especially tubers of potato, ginger, Jerusalem artichoke and chicory. Plant organs, such as hypocotyls, that are transiently and partly storage organs, can produce an active product. Cotyledons have shown a slighter secretion of active product.

In contrast, light, maybe through the photosynthetic process, was shown to inhibit this production from hypocotyls. In turn, plant leaves, either etiolated or not, did not produce such an active agent. Therefore the secretion of active product is organ-dependent. In a same plant, for instance chicory, roots secrete a more active product than leaves.

Beet roots, tubers of cassava, yam and sweet potato secrete less active products. Previously, it was reported that beets could display true cancer cells (Gaspar 1998; Gaspar *et al*. 1999). This could be related to a low ability to secrete an active product against tumour cells.

The physiological state of plant organ can modify the stress response. When sunflower hypocotyls are dark-grown (i.e. etiolated), they produce a secreted agent more active than light-grown hypocotyls. Breaking of dormancy process by low temperatures allowed the secretion of more active products by *H. tuberosus* tubers than by dormant tuber. Active products were related to presence of new proteins that are secreted after plant stress, that were seen by polyacrylamide gel electrophoresis. Active polypeptides have been characterized as a superoxide dismutase for *H. tuberosus* (Griffaut *et al.* 2007) and as a phospholipase A_2 for *S. tuberosum* (data not shown).

In conclusion, after an abiotic double stress, plants are able to secrete a protein complex that specifically inhibits the development of tumour (murine melanoma and crown-gall). Therefore, specific stimuli on plants can trigger a specific gene expression program, which would allow the production of new medicine by usually cultivated plants.

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Table 2 Plant secretion proteins.

Name	Molecular weights	Proteins[a]	References
Potato (*Solanum tuberosum*)	36000	PLA2-like	Data not shown
	16000 - 18000	Protein Inhibitors	Ledoigt *et al.* 2006
Jerusalem artichoke (*Helianthus tuberosus*)	28000	Alkaline Phosphatase	Griffaut *et al.* 2007
	18000	Superoxide dismutase	Griffaut *et al.* 2007
Chicory (*Cichorium intybus*)	16000	unknown	

[a] : NCBI blast data base

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