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Antimitotic effects of usnic acid on different biological systems

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Abstract. Usnic acid is a biosynthesis product characteristic of several epiphytic lichens such as *Evernia*, *Cladonia* and *Parmelia*. Usnic acid has several interesting biological properties. It is an antibiotic and it also seems to exert an antimitotic action. It has even been postulated that usnic acid can play a role as an environmental indicator, since its concentration varies according to the presence of toxic agents. A series of tests have been run on different biological systems such as fungi, yeasts, plant cells and neoplastic human cell cultures in order to make a general evaluation of the properties of usnic acid and to highlight any analogy between its effects on phylogenetically distant organisms. The results obtained confirm some of the already known properties of usnic acid and identify concentration ranges that are active against cells from different organisms. Furthermore, at low concentrations, the acid displays a capacity to stimulate cell metabolism in some of the biological systems tested.

Key words. Usnic acid; antimitotic and antimycotic properties; fungus; *N. tabacum*; yeast; tumour cell line; environmental indicator.

Usnic acid, which exists in nature in both the (+) and (−) forms, is the most abundant constituent of several lichen species such as *Usnea*, *Cladonia* and *Cetraria* [1]. Its antibacterial [2] and antimycotic properties, as well as its antimitotic activity against human neoplastic cell lines [3, 3a] are well known, and usnic acid has also recently been discovered to have antiviral properties (Y. Miura, personal communication). Relatively little is known about the activity of usnic acid on plant cells, although some authors have reported that it has the capacity to inhibit the development of some higher plants [4]. In vivo, usnic acid is probably produced by the lichens for defensive purposes and thus plays a role akin to that of the phytoalexins produced by plant organisms. The presence of polluting agents actually seems to stimulate the biosynthesis of usnic acid by the lichens, as indicated by environmental biomonitoring [5]. This suggests that usnic acid could be used for therapeutic purposes and as a biomarker by employing lichens that biosynthesize it as biological indicators of exposure. Its concentration in lichens would indeed seem to be related to the integral concentration of aromatic toxic agents [6]. The mechanism of action of this substance is still unknown, although a significant reduction in RNA content in rat liver treated with varying doses of usnic acid has been reported [7]. In all likelihood, this substance

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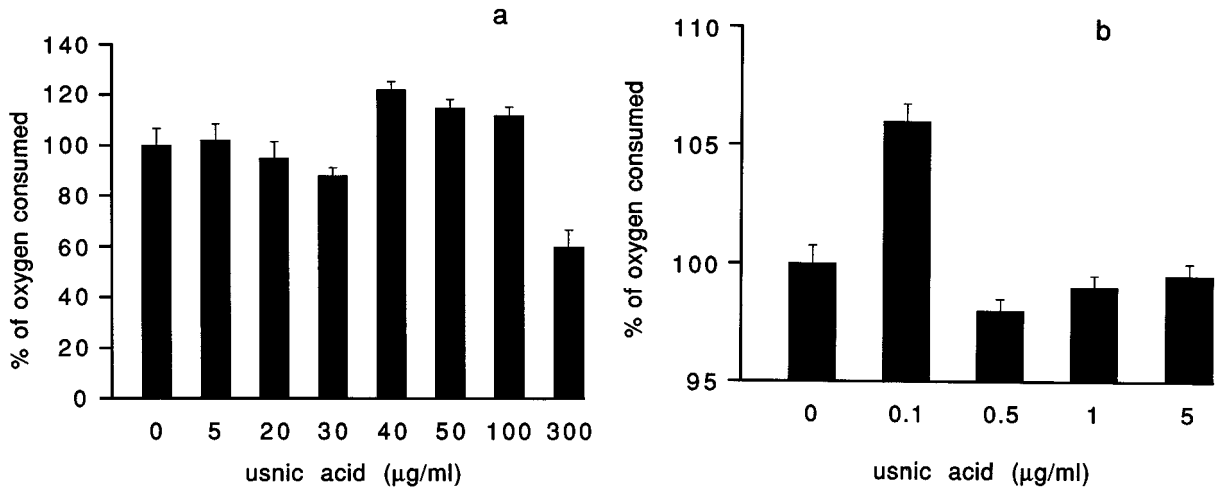

Figure 1. (*a*, *b*) Oxygen consumed in the presence of usnic acid.

acts by interfering with RNA synthesis. Despite its interesting properties and its therapeutic potential, the data reported in the literature on this substance are extremely fragmentary. Therefore, in order to make a general evaluation of the biological activity of usnic acid, we investigated its effects on a number of widely different biological systems. This led to interesting results concerning usnic acid's capacity to act as 1) an activator of the respiratory capacity of immobilized *Saccharomyces cerevisiae* yeasts, 2) a cytotoxic and anti-mitotic agent against mesophyll leaf protoplasts and cultured plant cells of *Nicotiana tabacum* (tobacco), 3) a cytotoxic and antimitotic agent against human tumour cells. The inhibition of growth of the parasitic fungi *Fusarium moniliforme* and of the germination of *Nicotiana tabacum* plantules has also been confirmed.

Materials and methods

Chemicals. All chemicals used for the experiments were commercial products of analytical grade. The (+) usnic acid >97% (DC) was from Fluka Biochemika and it was solubilized in distilled water by addition of NaOH up to a final pH 8.5 which proved to be nontoxic to *Saccharomyces cerevisiae*, cultured protoplasts, *Nicotiana tabacum* cells and seeds. For the tests on human tumour cells usnic acid was solubilized in DMSO, which proved to be nontoxic for the K-652, Ishikawa and HEC-50 cultures.

Respirometric tests. The *Saccharomyces cerevisiae* cells [8] were grown in a solid medium (2% glucose, 1% peptone, 1% agar and 1% yeast extract) for 48 h at 28 °C. One aliquot was removed using a spatula and placed in contact with the oxygen permeable membrane of a Clark electrode attached using a nylon net. The electrode was then immersed in a glycine buffer solution (pH 8) containing varying concentrations of usnic acid (from 0.1 to 300 µg/ml) for 8 min. It was then added to the 0.5% glucose solution and the amperometric electrode signal was detected using a potentiometer coupled to the electrode in order to measure the O_2 consumption in the absence and presence of glucose.

Cultured protoplasts. *Nicotiana tabacum* plants (SR1, Petite Havana variety) were grown in vitro in the growth chamber with a 16 h photoperiod. The protoplasts were prepared from plant leaves as previously described [9], and incubated at a concentration of 5×10^4 ml^{-1} in K3 medium, 0.4% glucose to which 1 mg/l NAA (2-naphtalenacetic acid) and 0.1 mg/l BAP (6-benzylaminopurine) were added at 26 °C in darkness in the presence and absence of usnic acid as shown in the figure legends. Cell viability was measured using fluorescein.

Tests on *Nicotiana tabacum* cells. Cultured *N. tabacum* cells were grown from stock for 5 days in MS basal medium saccharose 3% to which NAA 1 mg/l and kinetin 0.2 mg/l were added until the exponential growth phase. Usnic acid was then added at the concentrations shown in the legends to the figures and the samples to be used to measure the sediment value were taken after 3 days. Packed cell volume was measured after centrifugation at 200 × g for 5 min.

Tests on human tumour cells. The Ishikawa [10, 10a], K-562 [11] and HEC-50 [12] cells were cultured in flasks (3084 flasks – Falcon Plastic Co., Los Angeles, CA) in Minimum Essential Medium (Eagle's) with Earle's salts (MEM; GIBCO) containing 10% fetal bovine serum (FBS), 2% penicillin and streptomycin (PESS 7), 1% Fungizone. The cells were maintained in MEM plus 10% charcoal-treated FBS for at least 1 week before using

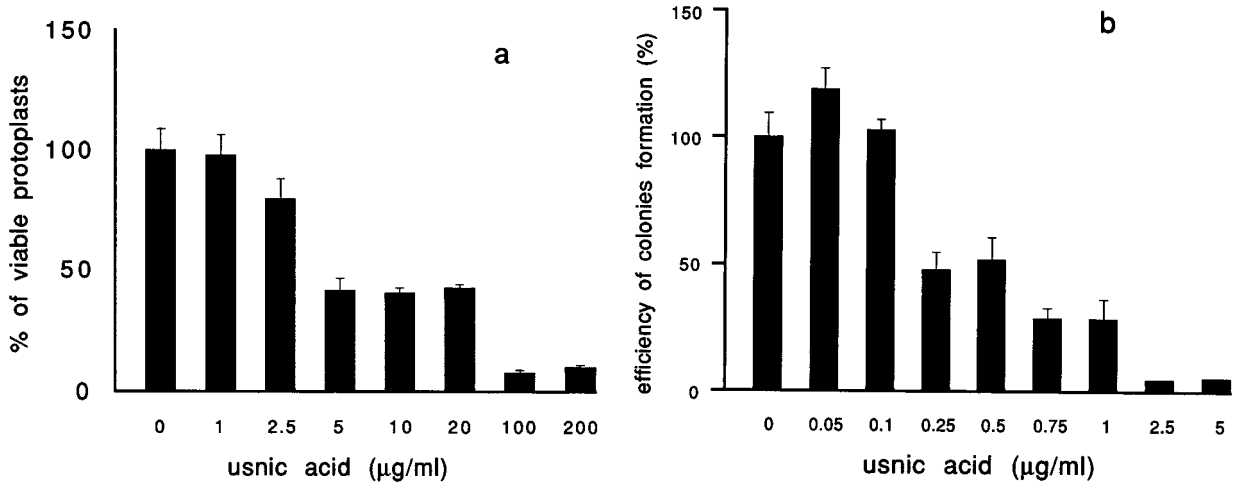

Figure 2. Effects of usnic acid on (*a*) *Nicotiana tabacum* protoplast viability, and (*b*) colonisation of protoplasts.

them for experiments. Cells were maintained at 37 °C in a humidified atmosphere of 95% air–5% CO_2.

Germination tests on *N. tabacum* seeds. Fifty seeds from SR1 plants for each concentration of usnic acid (see legends to fig. 4) were incubated in a 3% MS medium containing 10 g/l agar [13] in a growth chamber at 25 °C for 7 days. The size of the individual plantule was determined by analysis under the stereomicroscope.

Tests on *Fusarium moniliforme*. An agar cube containing mycelium and spores of the fungus *Fusarium moniliforme* was removed from a plate and inoculated onto 4 plates of media containing 8 g/l of NaCl, 10 g/l of tryptone, 5 g/l of yeast extract, 20 g/l of glucose and 2% agar, to which usnic acid was added or not, and at the concentrations shown in the legend (fig. 5). The plates were incubated for 6 days at a temperature of 25 °C and the diameter of the mycelium measured as a percentage of mycelium growth compared with controls.

Results

Effects of usnic acid on *Saccharomyces cerevisiae*. By determining oxygen consumption by the *Saccharomyces cerevisiae* cultures, the respirometric method used allowed yeast cell vitality in the presence of usnic acid to be measured and any toxic, stimulating or protective effect on growth to be evaluated. The results obtained are shown in figure 1. Concentrations of between 0 and 300 µg/ml reveal the absence of any toxic effect by usnic acid on yeast cell viability and indicate an effect of O_2 consumption for concentrations between 30 and 100 µg/ml (fig. 1a). The respirometric tests performed using low concentrations of usnic acid (from 0.1 to 5 µg/ml) reveal the absence of acute toxic effect of this substance as deduced from oxygen consumption of *Saccharomyces cerevisiae* cells, and also reveal a slight O_2 stimulation effect at concentrations lower than 0.5 µg/ml (fig. 1b).

Effects of usnic acid on leaf protoplasts of *N. tabacum*. Although several authors have reported a delaying or inhibiting action of usnic acid on the growth of higher plants, this substance's antimitotic action on plant cells has not been adequately investigated. Leaf mesophyll protoplasts cultured in the presence of varying doses of usnic acid were used. As soon as they were prepared, the protoplasts were incubated in the growth medium and hormones added in the presence and absence of usnic acid (from 1 to 200 µg/ml). The percentage of viable protoplasts was examined after 24 h by means of viable fluorescein staining (fig. 2a). Doses of

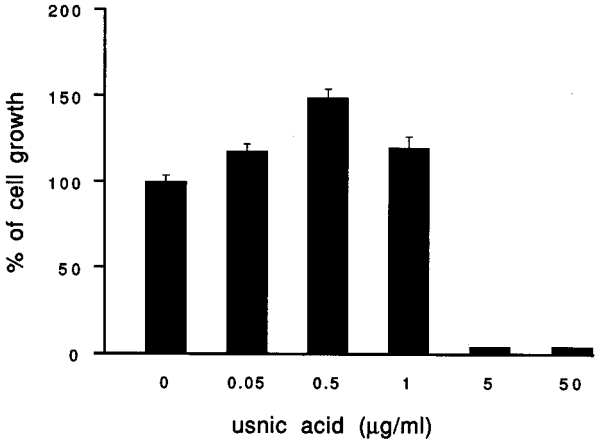

Figure 3. Effects of usnic acid on *Nicotiana tabacum* cell growth.

Table 1. Effects of usnic acid (50 μg/ml) on human tumour cells.

	K-562	Ishikawa	HEC-50
Control after 21 h	215 ± 23	202 ± 26	138 ± 20
+usnic acid	129 ± 18	76 ± 8	33 ± 8
Control after 46 h	531 ± 35	259 ± 30	338 ± 30
+usnic acid	54 ± 6	77 ± 6	51 ± 9*

*The reported data apply to the number of cells $\times 10^3$/ml.

Table 2. Effects of usnic acid (μg/ml) on *Fusarium moniliforme*.

Concentration of usnic acid (μg/ml)	Diameter (cm)	% MG
0	6.07	100%
1	5.52	90.93%
10	5.16	84.94%
100	2.96	48.82%

up to 2.5 μg/ml have little effect on protoplast viability (>80%), while concentrations of between 5 and 20 μg/ml reduced the number of viable protoplasts to about 40%. Higher doses (100–200 μg/ml) proved toxic for more than 90% of the cells. In view of these results the effect of doses of between 0.05 and 5 μg/ml on the proliferative capacity of cultured protoplasts was examined. A count was therefore made of the number of microcolonies formed by protoplasts exposed to different concentrations of usnic acid during incubation (fig. 2b). The results show that: 1) Usnic acid has a drastic inhibitory effect on protoplast proliferation even at very low concentrations which are not toxic for 90% of the cells. 2) The combined inhibitory and cytotoxic effect of the substance at slightly higher concentrations (2.5–5 μg/ml) completely inhibits protoplast proliferative capacity. 3) At a concentration 5 times smaller than that necessary to achieve partial inhibition of proliferation (0.05 μg/ml) usnic acid had a stimulating effect on protoplast proliferative capacity.

Effects of usnic acid on cultured tobacco cells. We investigated the effect of different doses of usnic acid on the cell division capacity of cultured *N. tabacum*. The concentrations tested were the same as those previously used for the protoplast tests. The experiments were performed on cells in the exponential phase that had been grown for 3 days in the presence of usnic acid. The results obtained are shown in figure 3. As can be seen in the histogram, the inhibition of cell growth occurs at doses about 5 times higher than those one required by protoplasts and becomes total at doses of 5 μg/ml. Furthermore, at doses lower than those at which inhibition occurs, a stimulation of cell division was observed. This effect was again achieved at doses 5–10 times higher than those one used for the protoplasts. A partial reversibility of the usnic acid effect was obtained using higher doses of between 0.05 and 1 μg/ml. These data (not shown) essentially confirmed those previously obtained on usnic acid's capacity to inhibit plant cell proliferation, and the discrepancies in the limiting concentration values can probably be accounted for by the fact that it is more difficult for the substance to penetrate the cell which has a wall.

Effects of usnic acid on human tumour cells. In order to test the anti-tumour properties of usnic acid on human cells, three different cell lines were used: 1) Stabilized K-652 leukemic cells as reference test (as data on the

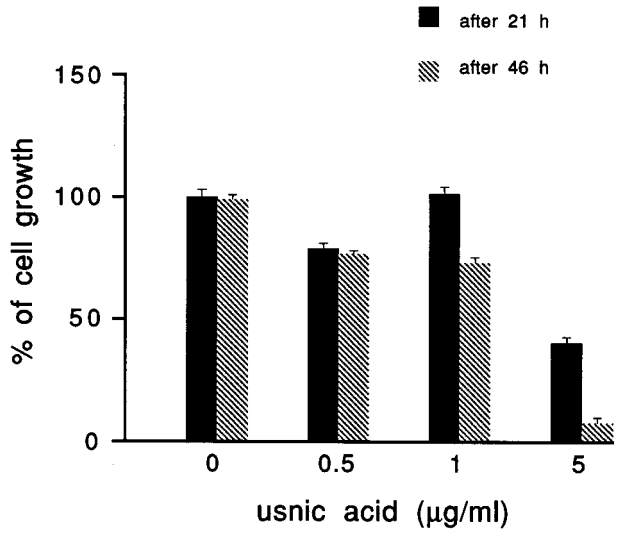

Figure 4. Effects of usnic acid on Ishikawa cells.

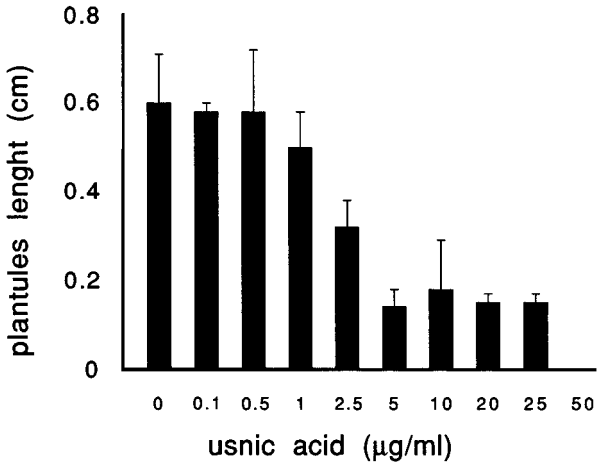

Figure 5. Effects of usnic acid on the germination of *Nicotiana tabacum* seeds.

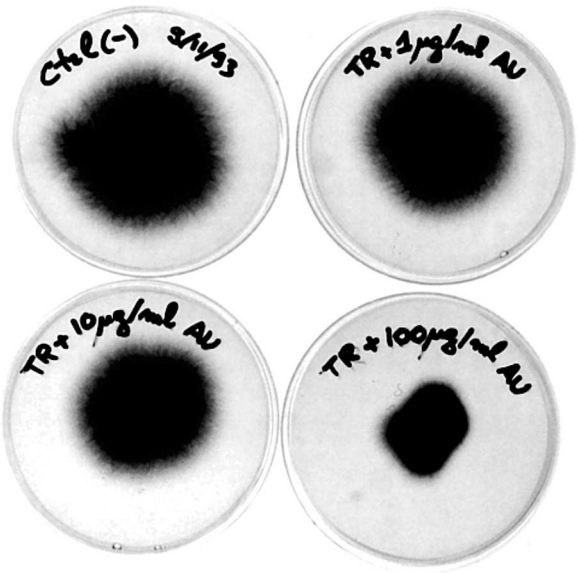

Figure 6. Effects of usnic acid on *Fusarium moniliforme*.

effect of usnic acid on these cells are available in the literature). 2) Stabilized monolayer Ishikawa human endometrial adenocarcinoma cells. This cell line, which has not yet been tested with this substance, responds to estrogens and contains receptors for estradiol and progesterone. 3) Stabilized monolayer HEC-50 human endometrial adenocarcinoma cells. This cell line, which has also never been grown in the presence of usnic acid, differs from the preceding one as it comes from histologically undifferentiated tissue and does not respond to estrogens. As shown in table 1, 50 µg/ml of usnic acid inhibit the proliferation of all the cell lines tested, albeit not totally (40–70%). The inhibiting capacity of usnic acid on these tumour cell lines does not only depend on the concentration of the substance but also on the length of time the cells remain in contact with the inhibitor. By increasing cell exposure to usnic acid up to 46 h, the percentage inhibition is increased to 70–90% (see table 1). Lower concentrations of usnic acid (from 0.5 to 5 µg/ml) were tested at different times for the Ishikawa cell line. The time effect was found to be significant for concentrations of 0.5–1 µg/ml, as shown in figure 4. The inhibitory effect at these concentrations occurs only after 46 h exposure. A concentration ten times lower than that shown in table 1 (5 µg/ml), with an exposure time of 46 h, is in any case sufficient to achieve practically total inhibition (90%) of the proliferation of this cell line (fig. 4).

Effects of usnic acid on the germination of *N. tabacum* seeds. The capacity of *N. tabacum* seeds to germinate in a culture medium containing usnic acid was analysed to determine the inhibiting effect of the latter substance, previously reported by other authors [2], on the early stages of growth of a organism. Fifty seeds for each usnic acid concentration used were incubated in MS medium containing the various inhibitor concentrations (from 0.1 to 50 µg/ml), and the length of the plantules obtained was measured after one week. As shown in the histogram in figure 5, a concentration of about 50 µg/ml was needed to inhibit germination completely. At concentrations of between 2.5 and 15 µg/ml the seeds germinate but the plantules suffer growth retardation, reaching 1/3 to 1/2 the length of the control plantules. Concentrations of less than 1 µg/ml have no inhibitory effect on either germination or plantule size. These results show that the effect of usnic acid is not limited to the proliferation of cells in cultures with or without walls, but that this substance can also interfere with the growth of the whole organism. The concentrations at which the substance acts are, as expected, comparable to those one used for cultured cells (from 5 to 50 µg/ml).

Effect of usnic acid on the growth of *Fusarium moniliforme*. The antimitotic properties of usnic acid were tested by means of growth assays using a *Fusarium moniliforme* mycelium, a necrotroph species capable of developing on a number of plants, both grasses and trees, including many Graminaceae. Usnic acid was added to the growth culture of the *Fusarium moniliforme* at concentrations from 1 to 100 µg/ml. As shown in table 2, a 9% growth inhibition is achieved at low usnic acid concentrations (1 µg/ml) (fig. 6). Higher concentrations of the substance lead to a gradual increase in inhibition of the fungus' growth, reaching a peak of 51% at a concentration of 100 µg/ml (fig. 6).

Discussion

The therapeutic properties of usnic acid (antibacterial, antimycotic and antimitotic against tumour cell lines) have been known for a long time and exploited in clinical practice. Furthermore, this substance also seems to exert a protective action on the toxic agents present in the atmosphere, since in lichens its concentration increases proportionally to the amount of the pollutants present. In this research a set of tests was run on different biological systems, to make a general evaluation of the properties of usnic acid and identify any analogy in the substance's effects on phylogenetically distant organisms. We cultured cell lines from plants (*N. tabacum*) and animals (leukemic neoplastic or adenocarcinoma cell lines) in order to test the antimitotic properties of usnic acid. *Saccharomyces cerevisiae* yeast was used to investigate the protective effect of the substance, the *Fusarium moniliforme* fungus to evaluate its antimycotic properties and germinating *N. tabacum* plantules to examine the inhibiting effect on the growth of a plant organism.

Interesting analogies were observed concerning the antimitotic capacity of usnic acid on cultured cells: proliferation was in fact inhibited at concentrations of 5–50 μg/ml in the case of both plant and animal cells provided that the duration of exposure was at least 46 h. The concentration may be 5 times lower if mesophyll leaf protoplasts are used, i.e. cells lacking the protective plant wall. This would seem to indicate that the effect exerted is influenced by the capacity of the substance to penetrate the cell. The experiments carried out on protoplasts also show that : A) a partial inhibitory effect on plant cell division takes place even at low concentrations values (less than 1 μg/ml, which is not toxic for 90% of cells), while proliferation is totally inhibited when, by increasing concentrations up to 2.5 μg/ml, the inhibitory effect is added to the cytotoxic effect. Although the mechanism of action of usnic acid is still unknown, the results obtained indicate that the substance acts in a similar way on cells of both animal and plant origin, probably by interfering with some key steps in cell division shared by such different cells. The experiments performed on *Saccharomyces cerevisiae* yeast, on the cells and protoplasts from *Nicotiana tabacum* and, to a much lesser extent, on human Ishikawa cells, also point to a positive effect of usnic acid in stimulating cell growth. This effect is strongly pronounced in the case of the respiratory activity of yeasts at concentrations of 30–100 μg/ml, (i.e. concentrations that are toxic in other experimental systems), and it is also present, although to a lesser extent, for concentrations around 0.1 μg/ml. The stimulating effect on cell and plant protoplast division takes place over the same concentration range as the less evident effect involving yeast cells (0.05–0.1 μg/ml) and at concentrations of 0.5–1 μg/ml in the case of human cell lines.

The usnic acid effect was also tested on germinating *N. tabacum* plantules and on *Fusarium moniliforme* fungus. The data obtained indicate that the inhibitory effect on plant cells also occurs in the organism as a whole. The inhibitory effect takes place over the same concentration range as that used for cultured cells (5–50 μg/ml) and becomes total and irreversible at 50 μg/ml. The plantules do germinate at low concentrations but display considerable growth retardation. Lastly, the antimycotic properties of the substance were demonstrated using the *Fusarium moniliforme* fungus, which is extremely harmful to rice and maize crops. The effect was not total and was obtained at high concentrations in the solid fungus medium. The growth of *Fusarium* is a highly complex matter due to the fungus' capacity to survive in the saprophytic state and to its frequent localization in histological sites practically inaccessible to pesticides. The possibility of using usnic acid to inhibit fungal growth is in any case dependent on the effects of the substance on infected plants.

Our results confirm the substance's antimitotic properties and furthermore identify a concentration range over which the acid expresses completely opposite properties, at least as far as some of the biological systems considered are concerned. It is our intention to continue the research in order to define the mechanism of action of this substance, although its capacity to act on such different systems suggests that its underlying mechanism is a simple one.

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