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SYSTEMIC ACQUIRED RESISTANCE IN SUNFLOWER (*Helianthus annuus* L.)

Systemic acquired resistance (SAR) to infection by Botrytis cinerea in the leaves of sunflower (Helianthus annuus L.) plants was induced following cotyledon inoculation with B. cinerea or treatment with abiotic inducers. Salicylic acid (SA), benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester (BTH), 2,6-dichloroisonicotinic acid (INA) or EDTA protected sunflower plants against Botrytis infection, that was revealed by a reduction in the number and area of the necrotic lesions in upper leaves after challenge inoculation with the pathogen. SA and BTH were more potent inducers than INA, EDTA or pre-inoculation with the fungus. In addition to resistance to B. cinerea, the upper leaves have also developed resistance to maceration by a mixture of cell wall-degrading enzymes. Calcium nitrate inhibited both the protective effect and the resistance of leaf discs to cell-wall degrading enzymes. All the tested chemicals increased the synthesis and excretion of sunflower phytoalexins — coumarins scopoletin and ayapin and induced the PR-proteins chitinase and 1,3-β-glucanase, being the inducer effect of each activator correlated with the level of protection against B. cinerea (BTH>SA>INA>EDTA). Thus, SAR induction is mediated by general increase of plant defence responses. This is the first report on SAR in sunflower.

Introduction. Acquired resistance is an inducible plant defence response triggered following infection with a necrotizing pathogen (either as a part of the hypersensitive response or as a symptom of disease) that confers protection against subsequent infection by that pathogen as well as a number of other potential bacterial, fungal or viral pathogens [1, 2]. The phenomenon is known since the begining of the last century [3], being reported in a number of dicot and monocot plant species; however its potential has not been evaluated yet in crops that like sunflower are of major economic importance. Induced resistance can be either local or systemic, thus, while local resistance is activated in the vicinity of the infected or wounded area, systemic acquired resistance (SAR) is distributed throughout the plant [4]. Recently there has been a great interest in SAR because the knowledge of the molecular basis that governs its induction can be very important in the development of genetically engineered crops with enhanced disease resistance. SAR can be induced not only after plant infection but also after plant treatment with elicitor preparations and a number of chemicals including the natural salicylic acid and some synthetic compounds (plant activators) like isonicotinic acid (INA) and benzothiadiazole (BTH) derivatives [2, 5]. Even though the acquired disease resistance is known since long, the molecular mechanism underlying this process is not completely understood. Research in this respect has been carried out using the experimental model plant systems like tobacco, cucumber and *Arabidopsis* and has been mainly focused at the role of salicylic acid and/or of hydrogen peroxide as endogenous signals for SAR induction [6, 7], and the correlation with the coordinate expression of defence genes (SAR genes) [8].

Sunflower (*Helianthus annuus* L.) is one of the most important oil-producing crops in the world. It is a host of a number of viruses, bacteria and fungi as well as parasitic weed species, some of them causing severe diseases and significant yield loses. In some cases (*Sclerotinia sclerotiorum* and *Orobanche cernua*) neither plant breeding programmes nor agrochemicals have completely provided full protection. Some of the classical plant defence responses like the induction of the multidefence coumarins scopoletin and ayapin and PRs have been previously documented in sunflower [9, 10]. This paper reports for the first time the induction of SAR in sunflower. Treatment of cotyledons with *Botrytis cinerea*, salicylic acid, benzothiadiazole, isoniconitic acid and EDTA protected sunflower plants against ulterior infection with *B. cinerea*.

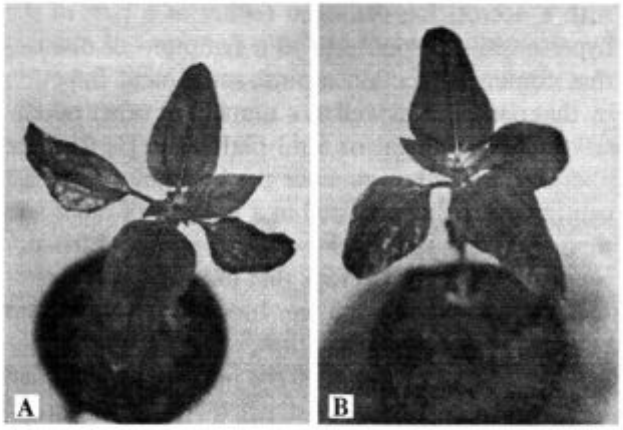

Fig. 1. The induction of SAR in sunflower (cv. Peredovick). Symptoms observed 5 to 7 days after challenge inoculation with *B. cinerea* (10^4 spores ml^{-1}) on the second upper leaves: **A** — control plants were cotyledon-treated with sterile distilled water; **B** — plants protected by BTH, 0.5 mM had restricted lesions covering 15 to 30 % of the leaf area

Material and Methods. *Plant material and fungal culture.* Seeds of the sunflower (*Helianthus annuus* L.) cultivar Peredovick were provided by Eurosemillas S.A. (Cordoba, Spain). The seeds were surface sterilized, germinated and grown in a growth chamber at 16-h photoperiod (photon flux density of 300 $\mu E\ m^{-2}\ s^{-1}$), 21/16 °C day/night-time temperature and 75–85 % relative humidity. Plants used for experiments were 16-days-old, corresponding to the two leaf pairs developmental stage (two expanded leaves and two young developing ones). *Botrytis cinerea* Pers.: Fr, isolate Bc-7, was obtained from infected tomato plants. For production of conidia, the isolate was subcultured in potato dextrose agar and incubated for 12–14 days at 21 °C in darkness.

Induction of SAR with fungi or abiotic inducers. For fungal inoculation, three 10 µl droplets of a conidial suspension (10^4 spores ml^{-1}) of *B. cinerea* were separately deposited on the upper surface of each cotyledon of 16-days old sunflower plants. Plants were placed in plastic bags at 21–22 °C for 48 h in order to provide the high relative humidity necessary for spore germination. For abiotic induction, three separate 10 µl drops of SA (7 mM), EDTA (5 mM), BTH (0.5 mM) or INA (0.03 mM) solutions (when necessary, pH was adjusted to 6.5) were applied to the surface of the cotyledons. Chemical treatments were repeated two more times on the 17th and 18th days. Control plants were treated with distilled water. In a set of plants and 24 h after the third treatment with the chemicals or 48 h after *B. cinerea* inoculation, cotyledons received an additional application of three separate 10 ml droplets of a 10 µM $Ca(NO_3)_2$ solution. Challenge inoculation was done 10, 17 and 24 days after cotyledon treatment by depositing on the upper surface of the first leaves three 10 µl droplets of a conidial suspension (10^4 spores ml^{-1}) of *B. cinerea*, while control plants received distilled water treatment. Plants were reincubated in moistened humidity plastic bags for 48 h. The used *B. cinerea* isolate caused brown necrotic lesions on both cotyledons and leaves, mainly located in the area where conidia droplets were deposited and visible 48–72 h after inoculation. Disease symptoms were scored by measuring the number and the area of the lesions and induced resistance was expressed as the percentage of the leaf-necrotized area with respect to the control. Values are the means of four replicates and all experiments were repeated twice.

Maceration of leaf tissue by cell wall degrading enzymes. Detached leaves were surface sterilized in a 10 % sodium hypochlorite solution and washed in sterile distilled water. Leaf discs (1 cm) were cut and placed into beakers containing 3 ml of 0.8 mM $CaCl_2$, vacuum infiltrated and pre-incubated for 24 h at 4 °C in the dark. Leaf discs were subsequently incubated for 24 h at 24 °C in the dark in a mixture of cell wall degrading enzymes containing 0.3 % cellulase Onozuka R-10 and 0.05 % macerozyme R-10, in 10 mM MES (pH 6.0), containing 0.2 M sucrose, 0.4 mM $CaCl_2$, 2 % PVP, 175 mM KCl and 22.5 mM $MgCl_2$. Leaf discs were carefully removed and the remaining solution was centrifuged at 1000 g for 10 min. The pellet was washed twice with 3 ml of protoplast medium (as above without the enzymes). The final pellet was redissolved in a minimum volume of the medium and the number of protoplasts was calculated. Control discs were incubated in sterile distilled water or with boiled enzymes. All samples were done in duplicate.

Coumarin extraction and analysis by TLC. Leaves of 45-days-old sunflower plants were treated with SA, BTH, INA, EDTA or sucrose by depositing on the upper surface 20 µl droplets of SA (7 mM), EDTA (5 mM), BTH (0.5 mM) or INA (0.03 mM) solutions and then incubated for 3 days at 23 °C in darkness under high humidity conditions. After this period, the droplets from three leaves (about 3 ml) were collected, coumarins were extracted by partitioning with ethyl acetate and analyzed by TLC as described [11].

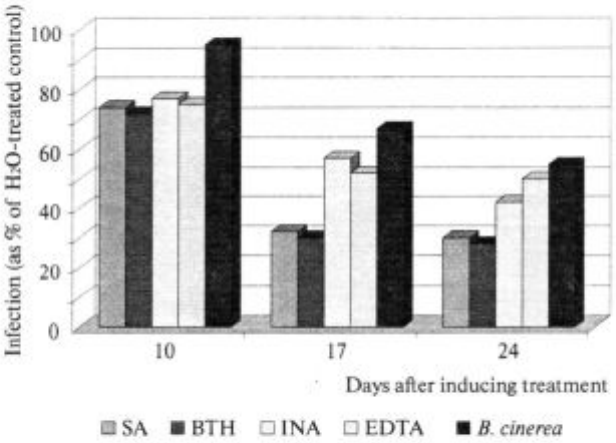

Fig. 2. SAR induction in upper leaves of sunflower challenged with *B. cinerea* at different time after cotelydon inoculation with the pathogen (10^4 spores ml^{-1}) or treatment with SA (7 mM), BTH (0.5 mM), INA (0.03 mM), EDTA (5 mM) or sterile distilled water. Development of disease was scored by determination of infected leaf areas of control plants were set to 100%, corresponding to X + Y %, indicate for days 10, 17 and 24. Each bar represents the means of four replicates, with the experiment repeated twice

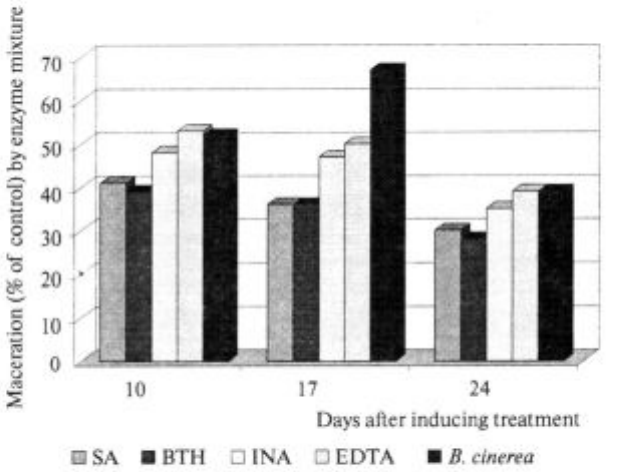

Fig. 3. Systemic induction of resistance to maceration by cell wall-degrading enzymes by abiotic elicitors and *B. cinerea* in upper leaves of sunflower (cv. Peredovick.)

Alternatively, leaf discs were incubated in Petri dishes with SA (7 mM), EDTA (5 mM), BTH (0.5 mM), INA (0.03 mM) or sucrose (0.1 M) solutions for 3 days at 23 °C in darkness. Coumarins were extracted from the liquid medium and analyzed by TLC [11].

Protein extraction and Western blot analyses. The first and the second leaves were collected at various intervals after treatment with abiotic inducers, frozen in liquid nitrogen and kept at −80 °C until used. Frozen leaves were ground with sand in the presence of 0.1 M Na-acetate buffer pH 5.2 (4.0 ml g^{-1} fresh weight). Homogenate was centrifuged at 10 000 g for 20 min and the supernatant was used for protein analysis by SDS-PAGE on 12.5 % polyacrylamide slab gel in the presence of 0.1 % SDS, being the amount of protein loaded of about 35 μg. For Western immunoblotting, the proteins contained in the gel were electrotransferred for 60 min at 200 V onto a nitrocellulose sheet (0.2 μm pore size) in a buffer containing 25 mM Tris, 192 mM glycine and 20 % methanol (pH 7.5). The blots were blocked in TBS/Tween buffer (10 mM TRis, 20 mM NaCl, 0.5% Tween 20, pH 8.0) containing 5 % defatted milk powder, and incubated with primary antibodies diluted in TBS/Tween containing 1 % BSA. Chitinase and β-1,3-glucanase were detected by using specific antisera against tomato 26 kD chitinase and 33 kD β-1,3-glucanase [12]. Antigens were visualized after incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad).

Results. Inoculation of sunflower leaves and cotyledons with spore suspension of *B. cinerea* caused necroses with lesions starting to be visible 48–72 h after inoculation, expanding in size during 5–7 days and becoming brown in colour due to the loss of pigments. Lesions were mainlly observed in the leaf/cotyledon area where droplets with the spore suspension were deposited (Fig. 1, A). Under the experimental conditions and with the isolate here utilized, the estimated necrosis area 7 days after inoculation was 80–90 %.

Treatment of the cotyledons with either spore suspension (10^4 spores ml^{-1}) or solution of SA (7 mM), BTH (0.5 mM), INA (0.03 mM) or EDTA (5 mM) prior to challenge inoculation 10, 17 or 24 days after, reduced the area and the number of the lesions in upper leaves (Fig. 1, B). This protective effect was more prominent in the second leaves, which expanded after induction, than in the first ones, which expanded before induction and when the challenge inoculation was done 24 days than 17 and 10 days after cotyledon treatment (Fig. 2). Reduction in the necrotizing tissue estimated when challenge inoculation was done 24 days after cotyledon induction was higher in BTH (72 %) and SA (68 %) than in INA (58 %), EDTA (48 %) or *B. cinerea* (42 %) treated plants. There was a difference between SA or EDTA and BTH or INA cotyledon treatments. While SA or EDTA caused tissue necrosis BTH or INA did not.

When leaf discs from systemically resistant leaves were incubated in a mixture of cell wall degrading

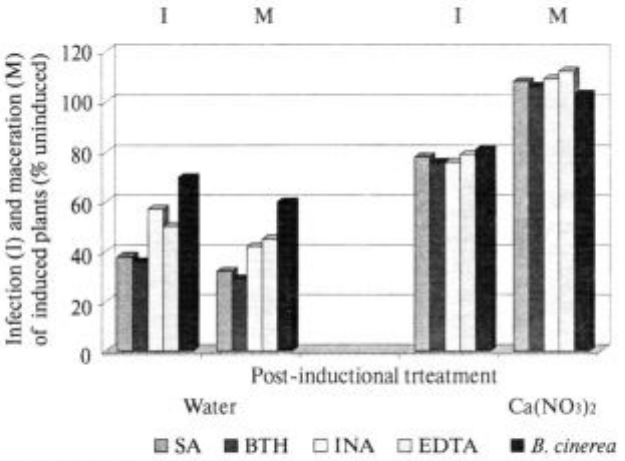

Fig. 4. *B. cinerea* infection and maceration by enzyme mixture of upper leaves of sunflower following treatment of cotyledons with inducing agents + calcium nitrate. Control plants were treated with water + 0.01 % Tween 20. Upper leaves were challenge inoculated with *B. cinerea* 10 days after treatment, and infection was assessed 7 days after challenge inoculation. Values are the means of four replicates

enzymes containing cellulase and macerozyme in an osmoticum solution, the number of released protoplasts was lower than the obtained from control, sterile distilled water treated, plants (Fig. 3). The number of released protoplasts ranged from about 40 % of the control for leaf discs from plants treated with SA or BTH to about 50 % of the control for leaf discs from plants treated with INA, EDTA or *B. cinerea*.

Application of calcium nitrate to the cotyledons after treatment with *B. cinerea* or abiotic inducers inhibited both the protective effect against *B. cinerea* and the resistance of leaf discs to maceration by cell wall degrading enzymes (Fig. 4). When challenge inoculation was done 17 days after cotyledon treatment values of the necrosed tissue were only 80 % of the control (water instead calcium nitrate treated cotyledons), with no differences between treatments. Similarly, the number of released protoplasts was much higher in calcium than in water treated (control) plants (Fig. 4). On the other hand, calcium nitrate when applied alone had no effect on *B. cinerea* infection in upper leaves (data not shown).

TLC analysis of drop diffusates and the medium of 45-days old sunflower leaves and leaf discs, treated with SA, BTH, INA or EDTA respectively, for 3 days revealed that all the chemicals induced the synthesis and excretion of sunflower phytoalexins — coumarins scopoletin and ayapin, with BTH and salicylic acid being the most potent inducers (Fig. 5) .

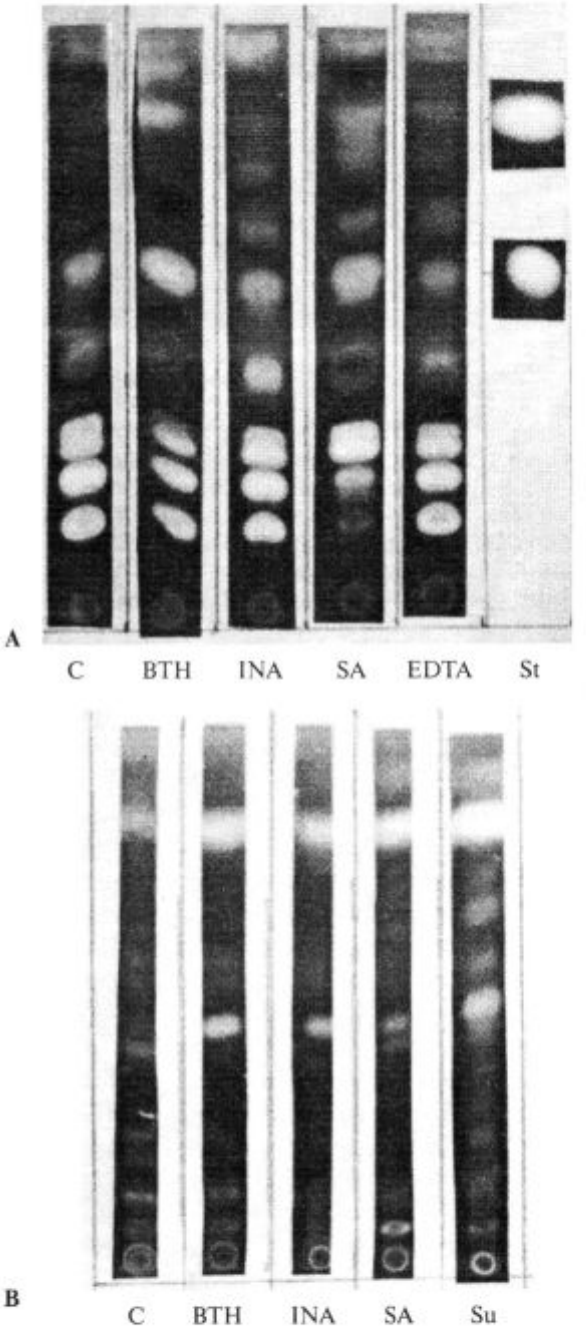

Fig. 5. Coumarin excretion in response to SAR inducers: **A** — Sunflower leaves were treated by depositing on the upper surface 20 µl droplets of BTH (0.5 mM), SA (7 mM), INA (0.03 mM), EDTA (5 mM) or sterile distilled water (control); **B** — Sunflower leaf discs were incubated with water or a solution of BTH (0.5 mM), SA (7 mM), INA (0.03 mM), EDTA (5 mM) or sucrose (0.1 M). Coumarins were extracted from the collected droplets or incubation medium by partitioning with ethylacetate and analyzed by TLC. C — control; Su — sucrose; St — standards

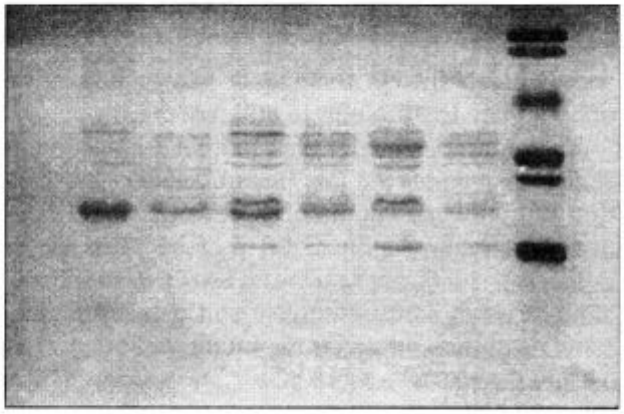

Fig. 6. SDS-PAGE analysis of proteins from leaf homogenates of sunflower (cv. Peredovick) following cotyledons treatment by abiotic inducers. Each line contains 35 μg of protein extracted from leaves collected 10 days following the inducing treatments. Molecular weight markers are shown at the right: 1 — control (water); 2 — KH_2PO_4, 10 mM; 3 — BTH, 0.5 mM; 4 — EDTA, 5 mM; 5 — SA, 7 mM; 6 — INA, 0.03 mM

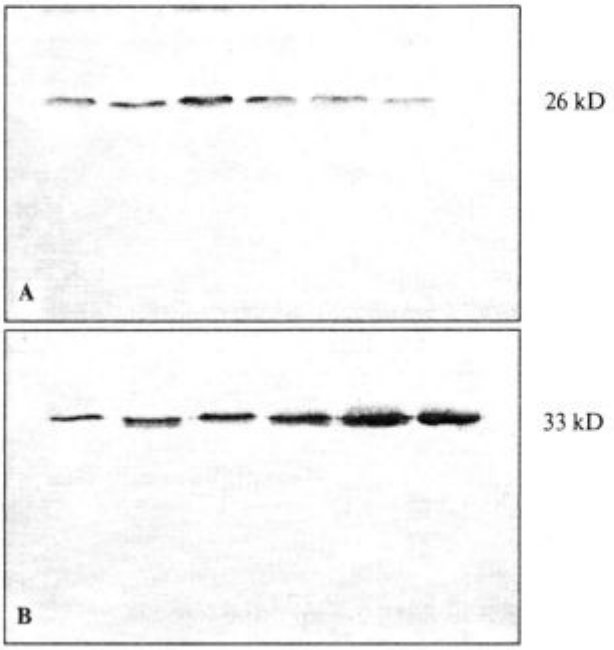

Fig. 7. Western blot analyses of sunflower chitinase and 1,3-β-glucanase in the upper leaves 10 days following cotyledons treatment. Panel **A** shows the accumulation pattern of the 26 kD chitinase and panel **B** shows the accumulation pattern of the 33 kD 1,3-β-glucanase. The treatments are the same as on Fig. 6

Electrophoretic analysis of total soluble proteins extracted at pH 5.2 from sunflower leaves (first and second pairs) 10 days after cotyledon treatment with abiotic inducers showed quantitative better than qualitative changes in the protein expression pattern, with a clear induction of at least six proteins with molecular mass ranged from 25 to 35 kD (Fig. 6). There were no differences between the treatments with the exception of a 30 kD protein band which accumulated to higher level after cotyledon treatment with salycilic acid. Changes in chitinase and 1,3-β-glucanase protein content after cotyledon treatment with abiotic inducers were analyzed by Western blot using specific antisera against tomato 26 kD chitinase and 33 kD 1,3-β-glucanase [12]. Both proteins were constitutively present, being induced in response to abiotic elicitors with not clear differences between treatments, with the exception of 1,3-β-glucanase which was detected a much higher amount after salicylic acid treatment (Fig. 7 A, B).

Discussion. As far as we know this is the first report on systemic acquired resistance in sunflower. In contrast to other plants, there is only a little knowledge about plant defense reactions in sunflower and almost nothing is known on defense genes activation. In this study on SAR induction in sunflower we have used a non-specific pathogen, *Botrytis cinerea*, that causes necrotic lesions in plant tissue. Protection against leaf infection by *B. cinerea* is enhanced by a prior cotyledon inoculation with the pathogen and treatments with natural SA or synthetic BTH or INA, chemicals which had been shown to be very efficient in SAR induction in other plant systems [2, 5, 13]. SAR induction by EDTA has been reported in a limited number of plants like cucumber, being effective in the protection against *Colletotrichum lagenarium* [14]. Interestingly and different from our results, SAR induction did not effectively protect tobacco plants against *B. cinerea* [8], indicating differences between plant systems as refers to SAR induction against specific pathogens.

The cotyledon treatment procedure used for SAR induction and the period of 10 to 24 days before challenge inoculation was optimized. That was selected on base of our interest in sunflower downy mildew and broomrape parasitism and taking into account that succesful infection by the fungus and parasite occur during early period of sunflower growth. The importance of three successive inducing treatments with abiotic inducers should be emphasized. In previous unsuccessful attempts to induce SAR, with for example SA, only single treatments with lower concentrations were used [15].

In our system SA and BTH were more potent SAR inducers than INA, EDTA or *B. cinerea* and for all of them the protective effect was more prominent in the second leaves, which expanded after induction, than in the first ones, which expanded before induction and when challenge inoculation was done 24 days than 17 or 10 days after cotyledon treatment. Although it has been proposed that SA, BTH and INA induce SAR via the same signal transduction pathway [16], in sunflower there are differences between inducing treatments: while SA and EDTA treatment caused tissue necroses, INA or BTH did not.

Walters and Murray [17] showed that induction of resistance to rust infection in broad bean by EDTA and phosphate was inhibited by calcium nitrate. We have obtained in our sunflower system a similar inhibitory effect by calcium independently of the inducer, supporting the idea of a similar mechanism for biotic and abiotic SAR induction. The involvement of calcium in the defence reactions signal transduction pathway has been documented [18, 19], although its mode of action remains unclear.

SAR is associated with the expression of a set of genes called SAR genes [20], some of them, but not all, being defensive genes. In our system SAR induction in non-infected sunflower leaves is accompanied by an increase of such defence reactions as: cell wall reinforcement, phytoalexin and PR-proteins synthesis. The level of resistance to *B. cinerea* in leaves after cotyledon treatment by different chemicals is well correlated with resistance to cell wall degrading enzymes and the induction of phytoalexins and the PR-proteins (chitinase and 1,3-β-glucanase). These results suggest that SAR induction against *Botrytis* operates, at least in part, by activating processes that would prevent pathogen infection, either by inhibiting spore germination or tissue penetration. It has been reported that sunflower coumarins scopoletin and ayapin are multidefence inducible compounds, being excreted better than accumulated in plant tissue [21, 22]. We pressume that production and excretion of scopoletin and ayapin in sunflower is part of an extracellular first defence line against pathogen infection. Both coumarins inhibit *B. cinerea* spore germination (our unpublished data), an effect previously described [23].

Induction of chitinases and 1,3-β-glucanases in sunflower in response to physical and chemical stress factors has been previously reported [24]. In our work we have shown that SAR in sunflower leaves is accompanied by the constitutive expression of 26 kD chitinase and 33 kD 1,3-β-glucanase. Cotyledon treatment with abiotic elicitors induced an accumulation of both these PR-proteins to different extent.

The results obtained suggest that SAR induction in sunflower requires the first line of defence — excretion of antifungal coumarins scopoletin and ayapin but also accumulation of PR-proteins. The use of inducers in sunflower to activate SAR provides novel alternatives for disease control and detailed understanding of this pathway is important for both practical and theoretical reasons.

РЕЗЮМЕ. Системную устойчивость (СУ) в листьях подсолнечника (*Helianthus annuus* L.) к заражению фитопатогенным грибом *Botrytis cinerea* индуцировали с помощью предварительной инокуляции семядолей спорами этого гриба или их обработкой абиотическими элиситорами. Салициловая кислота (СК), бензо-(1,2,3)-тиадиазол-7-карботио-S-метиловый эфир (БТН), 2,6-дихлоризоникотиновая кислота (ИНК) или ЭДТА защищали растения подсолнечника от заражения *Botrytis*. Индуцирование СУ регистрировали по уменьшению количества и размеров некрозов в верхних листьях после искусственного их инфицирования. СК и БТН оказались более эффективными элиситорами, чем ИНК и ЭДТА или пре-инокуляция спорами гриба. Кроме повышенной устойчивости к заражению *B. cinerea*, у верхних листьев отмечено повышение устойчивости к мацерации смесью ферментов, деградирующих клеточную стенку. Нитрат кальция подавлял защитный эффект и устойчивость к смеси ферментов. Все испытанные элиситоры вызывали усиление синтеза и экскреции фитоалексинов подсолнечника — кумаринов скополетина и ауапина, а также образование патогензависимых (PR) белков, хитиназы и 1,3-β-глюканазы. При этом индуцирующая активность абиотических элиситоров коррелировала с их способностью защищать листья от *B. cinerea* (БТН > СК > ИНК > ЭДТА). Следовательно, индукция СУ у растений связана с активацией защитных реакций. Это первое сообщение об индуцировании СУ у подсолнечника.

РЕЗЮМЕ. Системну стійкість (СС) у листя соняшника (*Helianthus annuus* L.) до ураження фітопатогенним грибом *Botrytis cinerea* індукували за допомогою попередньої інокуляції сім'ядолей спорами цього гриба або їх обробкою абіотичними еліситорами. Саліцилова кислота (СК), бензо-(1,2,3)-тіадиазол-7-карботіо-S-метиловий ефір (БТН), 2,6-дихлорізонікотинова кислота (ІНК) або ЕДТА захищали рослини соняшника від ураження *Botrytis*. Индукування СС реєстрували за зменшенням кількості та розміру некрозів у верхньому листі після штучного їх інфікування. СК та БТН виявились більш ефективними еліситорами, ніж ІНК та ЕДТА або пре-інокуляція спорами гриба. Крім підвищеної стійкості до ураження *B. cinerea*, у верхньому листі відзначено підвищення стійкості до мацерації сумішшю ферментів, які деградують клітинну стінку. Нітрат кальцію пригнічував захисний ефект та стій-

кість до суміші ферментів. Все досліджені еліситори викликали підсилення синтезу и екскреції фітоалексинів соняшника — кумаринів скополетину та ауапіну, а також утворення патогензалежних (PR) білків, хітинази и 1,3-β-глюканази. При цьому индукуюча активність абіотичних еліситорів корелювала з їх здатністю захищати листя від *B. cinerea* (ВТН > СК > ІНК > ЕДТА). Отже, індукція СС у рослин пов'язана з активацією захисних реакцій. Це перше повідомлення про индукування СС у соняшника.

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