

Effect of Silymarin on Plant Tissue Cultures

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Abstract. The effect of silymarin complex on various types of explants differing in their nutrition requirements was investigated. The growth of tumorous periwinkle (*Catharanthus roseus* [L.] G. DON) callus tissue was still identical with the control tissue at the silymarin concentration of 35 mg in 1000 ml of the nutrient medium. However, this silymarin concentration totally inhibited the growth of habituated periwinkle callus tissue; in the presence of 10 mg of silymarin, the growth of this tissue was similar to that of the corresponding tissue grown without silymarin. The growth of tobacco callus tissue (D-strain) requiring for its growth kinetin was reduced by 46.2% at the concentration 10 mg of silymarin in 1000 ml of nutrient medium, but its dry weight was increased by 21% in comparison with the control. Silymarin was most effective on the growth of callus derived from tobacco (*Nicotiana glauca* GRAEB.) stem pieces; callogenesis was observed in control tissue in 89.5% cases while in the presence of silymarin (10 mg) only in 48.6%. The primary callus growth was strongly inhibited, too (by 89.9%). The organogenesis onset was never observed on tobacco stem pieces cultured on a nutrient medium with kinetin and IAA in the presence of silymarin. When all types of explants were transferred from the medium with silymarin on control medium, normal growth appeared very soon and the differences between the experimental and control explants were smoothed out during two months. These results indicate that the observed changes might be due to the blocking of membrane system permeability leading to an insufficient supply of cells with nutrients and growth substances.

The term silymarin or silymarin complex involves an active group of compounds isolated from the drug plant *Silybum marianum* (L.) GAERTN. The most important components of this complex are the isomers silybin (the main compound previously designated as silymarin), silydianin and silychristin and additionally their dehydrocompounds and various oligomers and simple flavonoids in minute proportion (WAGNER *et al.* 1974). PELTER and HÄNSEL (1968) proposed to indicate these conjugates flavolignans but some authors do not agree with this denomination and prefer the original term silymarin, which at present means the whole active complex of these conjugates (WAGNER *et al.* 1974).

Silymarin has an antihepatotoxic effect (*e.g.* FASSATI and FASSATI 1973, RAUEN and SCHRIEWER 1973) and operates as a membrane stabilizator (HAGERS Handbuch 1979). It is supposed that its action consists in the mem-

Received July 2, 1982; accepted November 1, 1982

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brane effect (SCHRIEWER and RAUEN 1973a); silymarin functions both protectively and curatively in organelles (*e.g.* mitochondria) but the mechanism of this action is not yet clear (SCHRIEWER and RAUEN 1973b). BACHNER-JASCHKE and KOCH (1979) studied *in vitro* the coupling of silymarin components on plasmatic proteins and synthetic polymers and infer from the results of their experiments that the membranes do not absorb any essential silymarin amounts. SONNENBICHLER *et al.* (1980) investigated the distribution of silybin labelled with tritium in rat livers and found on autoradiographs an accumulation of the flavolignan compounds in cytosol cell fraction and on nuclear membranes. Silymarin administered to the patients over long periods at high doses does not produce any toxic side effects, and for this reason many commercial preparations are manufactured *e.g.* Legalon, Hepadestal, Cardomarin, Silliver *etc.* (NEGWEGER 1978, Index Nominum 1980, 1979).

The aim of our study was to investigate the silymarin effect on plant cells. As far as we know this question has not yet been studied. We made our experiments with plant tissues cultured *in vitro*. The explants present simplified and less specialized systems and their growth is fully dependent on the material supply from the nutrient medium. If the nutrients from the outside medium do not penetrate into the plant cells for any reason, the tissues do not grow and the explants remain in the phase of rest or "dormancy" which is after some time overcome or the tissues die. From the phytopathological point of view, especially when the explants are used for freeing the plants from their diseases, the prolongation of the rest phase might be according to our experiences (PETRŮ and ULRYCHOVÁ 1978) very important for the elimination of the pathogen. The application of a substance which is supposed to have a negative effect on membrane system permeability might lead to a purposeful prolongation of the rest phase with the aim to get rid of the infectious agent in the corresponding explant. These were the theoretical considerations preceding our investigation of the silymarin effect on plant tissues.

MATERIAL AND METHODS

The silymarin effect was studied on various types of explants differing by its nutrient requirements. The first one was the tumorous *Catharanthus roseus* (L.) G. DON callus tissue (BRAUN 1961). The second explant was callus tissue derived from a healthy plant of the same species. Both tissues were obtained from Prof. Braun from the Rockefeller Institute in New York twenty years ago and since that time they have been regularly passaged in our laboratory. The third type was tobacco callus tissue (D-strain) derived from tobacco pith (*Nicotiana tabacum* L. cv. Wisconsin 38). This tissue grows on a synthetic medium only in the presence of cytokinins (*e.g.* kinetin) and was obtained from Ing. Kamínek, CSc. from the Institute of Experimental Botany, Czechoslovak Academy of Sciences. The following type of explant were stem pieces of tobacco (*Nicotiana glauca* GRAH.); the formation of primary callus and the onset of organogenesis were observed on these tissues.

The first two types of callus tissue were cultured on a modified medium according to WHITE (1943); the concentration of macronutrients was doubled and pyridoxin was omitted.

Tobacco callus tissue (D-strain) was cultured on a modified Murashige and Skoog medium (PETRŮ and ULRYCHOVÁ 1975).

The last mentioned nutrient medium was also used for the cultivation of the primary callus tissue from the tobacco (*N. glauca* GRAH.) stem; besides, this explant was cultured on a Murashige and Skoog medium containing 2,4-D (ULRYCHOVÁ and PETRŮ 1975).

All nutrient media were solidified by Bacto-Agar Difco (0.8%).

Silymarin was added to the corresponding media after solution in 96% ethanol. We assayed the range of available concentrations in preliminary experiments and applied this substance within 1 to 35 mg in 1000 ml of nutrient medium.

Callus tissue cultures were established by aseptic passage of callus pieces weighing 70 to 80 mg. Ten Erlenmeyer flasks (100 ml) containing 50 ml of nutrient media were in each experimental group. Every flask contained two pieces of callus tissue. The cultivation was carried out in the dark at the temperature 25 °C. The callus growth was evaluated by weighing the tissue after 4 to 8 weeks.

Studying the silymarin effect on the formation of primary callus and onset of organogenesis we transferred two 3 mm long stem pieces of tobacco (*N. glauca* GRAH.) on the corresponding nutrient media. The last mentioned types of explants were observed in two repeated series established in April and May.

RESULTS AND DISCUSSION

The highest concentration of silymarin *i.e.* 35 mg in 1000 ml of nutrient medium was applied in experiments with tumorous callus tissue; this dose corresponds to one coated tablet of Legalon. Lower concentrations were without effect. The tumorous tissue originates by irreversible transformation of normal plant cells initiated by a tumor-inducing principle (TIP), produced by a specific bacterium known as *Agrobacterium tumefaciens*. The fully autonomous tumor cells differ from the normal counterparts by its ability to synthesize essential metabolites as IAA and kinetin and by the change in membrane permeability or ion-transport system (BRAUN 1961). The concentration 35 mg of silymarin did not inhibit the growth of tumorous callus tissue and only a slight pH shift took place inside the callus and in the nutrient medium in comparison with the control tumorous tissue cultured without silymarin. In the control tissue, pH slightly decreased in both the callus and the medium during cultivation. The pH of the callus did not change in the experimental variant with silymarin, but its value in the nutrient medium decreased more expressively (from the value 5.94 to 5.22).

The second type of callus tissue — *i.e.* the tissue derived from a healthy periwinkle plant — was much more sensitive than the preceding one. The highest applicable silymarin concentration was 10 mg in 1000 ml. At a higher concentration, the growth of this tissue stopped. This type of tissue called "habituated" resembles partially the tumorous one in the ability to synthesize essential metabolites but at a decreased rate. The silymarin concentration not inhibiting the tumorous callus tissue growth suppressed fully the growth of the habituated tissue and it was necessary to reduce the concentration to less than one third.

The concentration 10 mg of silymarin in 1000 ml of nutrient medium inhibited the growth of the tobacco callus tissue (D-strain) requiring for its growth kinetin by 46.2% in comparison with the control. The average callus

weight was in the control $1.43 \pm 3 \times 0.12$ g and in the experimental tissue with silymarin $0.77 \pm 3 \times 0.06$ g. This difference was highly significant, $P < 10^{-7}$. On the contrary, the percentage of the dry weight in the experimental tissue with silymarin was increased by 21% in comparison with the control. The average value of the dry weight of the control was 5.32% and of the experimental tissue 6.45%; this difference was significant too, $P < 0.01$.

The most expressive inhibiting effect of silymarin was observed in explants established from tobacco (*N. glauca* GRAH.) stem pieces on both nutrient media. On the medium with 2,4-D, callogenesis set on in 34 stem pieces from the total number of 38 (*i.e.* 89.5%) in the control, while in the experimental variant with silymarin (10 mg in 1000 ml), only on 18 of 37 stem pieces (*i.e.* 48.6%). The growth of the primary callus was strongly inhibited in the presence of silymarin, on the average by 89.9 % (Fig. 1).

Similar inhibiting effect of silymarin was proved on tobacco stem pieces cultured on medium supplemented with kinetin and IAA. The organogenesis onset occurred on the control explants after six to eight weeks but in the cultures with silymarin the formation of shoots did not start even after six months (Fig. 2).

When the plant tissues of all investigated types were transferred from the experimental medium with silymarin to the corresponding control medium after one to three months, they started to grow normally very soon and the differences between the control and experiment were smoothed away in the majority of cases. Only primary cultures mostly died after a prolonged period (6 months) when maintained on the medium with silymarin. The changes determined in various tissue types, will it be the pH change, decreased callus growth and the callogenesis and organogenesis suppression in primary cultures, lead to the conclusion that these changes might be due to the blocking of the membrane system permeability bringing about insufficient nutrient and growth substance supply into the cells. This supposition is in agreement with the fact that the tissues that do not require exogenous supply of growth substances and are supposed to have a changed membrane permeability (tumorous and habituated tissues), supported considerably higher silymarin doses than *e.g.* tobacco callus tissue (D-strain) requiring kinetin. The most conspicuous silymarin effect was observed in explants derived from tobacco stem pieces, which are totally dependent on exogenous nutrients and growth substances.

Our results providing indirect evidence of a negative influence of silymarin on membrane system permeability of plant cells might be utilized for the regulation of the rest phase length of the explants leading to the pathogen elimination (PETRŮ and ULRYCHOVÁ 1978), and thus outline new possibilities in improvement processes in plant tissue cultures.

Acknowledgement

The authors wish to express their sincere appreciation to RNDr. PhMr. Miroslav Karmazín, CSc. from the State Drug Control Institute in Prague for supplying the pure silymarin substance.

REFERENCES

- BACHNER-JASCHKE, I., KOCH, H.: Die Bindung der Silymarin-Substanzen an Plasmaproteine und synthetische Polymere. — Arch. Pharm. (Weinheim) 312 : 954–958, 1979.
- BRAUN, A. C.: Plant tumors as an experimental model. — Harvey Lectures 56. Pp. 191–210. Academic Press, New York 1961.

- FASSATI, P., FASSATI, M.: [Silymarin and some problems of present therapy of chronic hepatopathies.] In Czech. — Čas. Lék. čes. **112** : 865—869, 1973.
- HAGERS Handbuch der pharmazeutischen Praxis. VI. Bd. Silybum. Pp. 398—404. Springer Verlag, Berlin—Heidelberg—New York 1979.
- Index Nominum 1980. Pp. 913. Centre Scientifique de la Société suisse de Pharmacie. Zurich 1979.
- NEGWEGER, M.: Organisch-chemische Arzneimittel und ihre Synonyma. — Academie-Verlag, Berlin 1978.
- PELTER, A., HÄNSEL, R.: The structure of silybin (Silybum substance E_c), the first flavonolignan. — Tetrahedron Lett. **1968** : 2911—2916, 1968.
- PETRŮ, E., ULRYCHOVÁ, M.: Persistence and spread of mycoplasma in axenic callus tissue cultures of tobacco (*Nicotiana glauca* GRAH.) in the presence of kinetin and IAA in nutrient medium. — Biol. Plant. **17** : 352—356, 1975.
- PETRŮ, E., ULRYCHOVÁ, M.: Behaviour of MLO evoking potato witches' broom in callus tissue culture of *Solanum laciniatum* AIT. and *Nicotiana tabacum* L. cv. Samsun. — Biol. Plant. **20** : 383—386, 1978.
- RAUEN, H. M., SCHRIEWER, H.: Die antihepatotoxische Wirkung von parenteral verabreichtem Silymarin bei der Leberschädigung der Ratte durch CCl₄. — Arzneim. Forsch. **23** : 148—149, 1973.
- SCHRIEWER, H., RAUEN, H. M.: Die Wirkung des Silymarins auf das fremdstoffmetabolisierende Enzymsystem der intakten Ratten. — Arzneim. Forsch. **23** : 155, 1973a.
- SCHRIEWER, H., RAUEN, H. M.: Die antihepatotoxische Wirkung von parenteral verabreichtem Silymarin bei der Galaktosamin-Hepatitis der Ratte. — Arzneim. Forsch. **23** : 159, 1973b.
- SONNENBICHLER, J., MATTERSBERGER, J., HANSEN, G.: Untersuchungen zum Wirkungsmechanismus von Silybin, III. Aufnahme des Flavolignans Silybin in Rattenleberzellen. — Hoppe-Seyler's Z. physiol. Chem. **361** : 1751—1756, 1980.
- ULRYCHOVÁ, M., PETRŮ, E.: Elimination of mycoplasma in tobacco callus tissue (*Nicotiana glauca* GRAH.) cultured *in vitro* in the presence of 2,4-D in nutrient medium. — Biol. Plant. **17** : 103—108, 1975.
- WAGNER, H., DIESEL, P., SEITZ, M.: Zur Chemie und Analytik von Silymarin aus *Silybum marianum* GAERTN. — Arzneim. Forsch. **24** : 466—471, 1974.
- WHITE, P. R.: A Handbook of Plant Tissue Culture. — The Jacques Cattell Press, Lancaster, Pennsylvania 1943.

Figures at the end of the issue.

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SILYMARIN AND PLANT TISSUE CULTURES

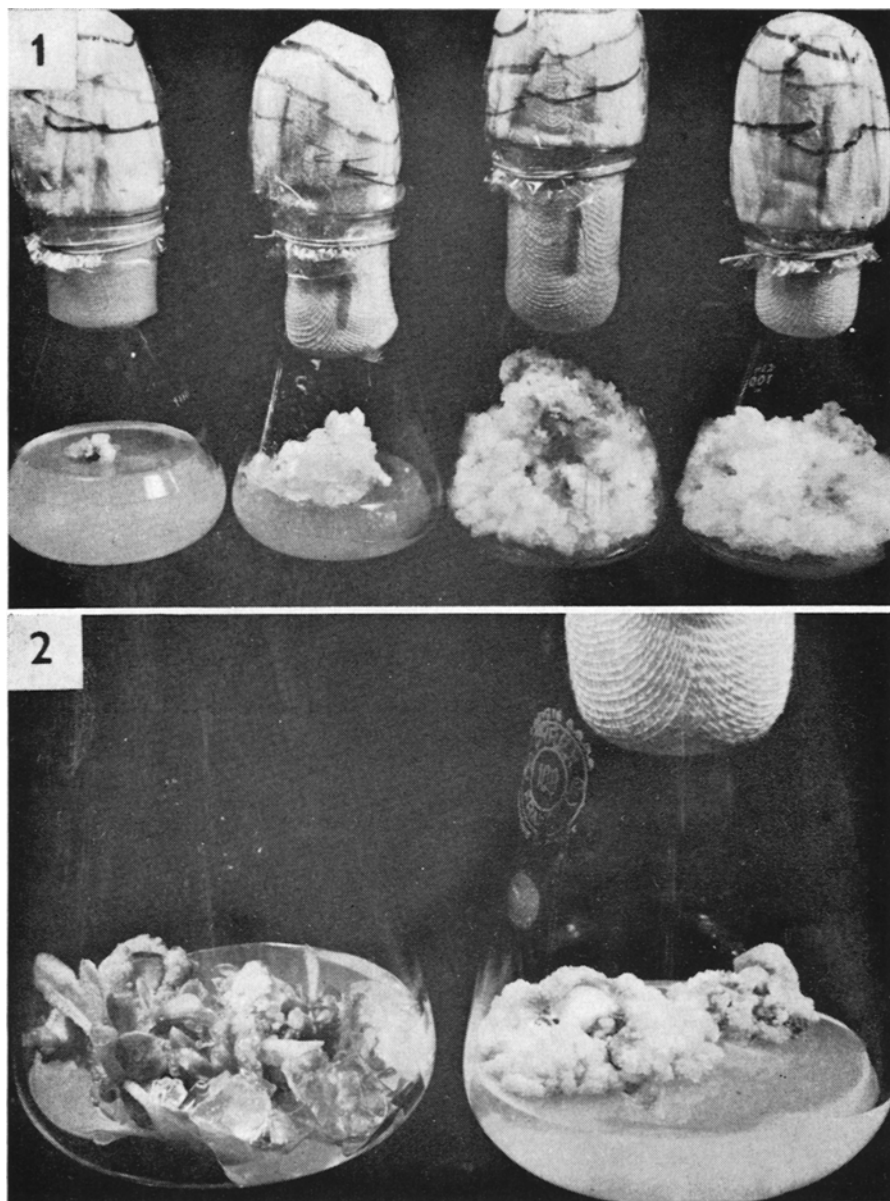


Fig. 1. Primary callus formation on tobacco stem pieces (*Nicotiana glauca* GRAH.) in the presence of silymarin (10 mg in 1000 ml of nutrient medium) ,three months after the culture establishment; two flasks on the left — experiment, two flasks on the right — control.

Fig. 2. Organogenesis on two tobacco stem pieces (*Nicotiana glauca* GRAH.) on the control medium (at the left) and on a medium supplemented with 10 mg of silymarin (on the right), three months after the culture establishment.