

## Callus culture for biomass production of milk thistle as a potential source of milk clotting peptidases

### Cecilia Cimino

Laboratorio de Investigación de Proteínas Vegetales  
Universidad Nacional de La Plata  
La Plata 47 y 115, CC 711, 1900  
La Plata, Argentina  
Tel: 54 221 4235333 ext. 57  
Fax: 54 221 422 4067  
E-mail: [cvcimino@biol.unlp.edu.ar](mailto:cvcimino@biol.unlp.edu.ar)

### Sandra Vairo Cavalli

Laboratorio de Investigación de Proteínas Vegetales  
Universidad Nacional de La Plata  
La Plata 47 y 115, CC 711, 1900  
La Plata, Argentina  
Tel: 54 221 4235333 ext. 57  
Fax: 54 221 422 4067  
E-mail: [svairo@biol.unlp.edu.ar](mailto:svairo@biol.unlp.edu.ar)

### Francisco Spina

Laboratorio de Investigación de Proteínas Vegetales  
Universidad Nacional de La Plata  
La Plata 47 y 115, CC 711, 1900  
La Plata, Argentina  
Tel: 54 221 4235333 ext. 57  
Fax: 54 221 422 4067  
E-mail: [frankspina1@hotmail.com](mailto:frankspina1@hotmail.com)

### Claudia Natalucci

Laboratorio de Investigación de Proteínas Vegetales  
Universidad Nacional de La Plata  
La Plata 47 y 115, CC 711, 1900  
La Plata, Argentina  
Tel: 54 221 4235333 ext. 57  
Fax: 54 221 422 4067  
E-mail: [natalucci@biol.unlp.edu.ar](mailto:natalucci@biol.unlp.edu.ar)

### Nora Priolo\*

Laboratorio de Investigación de Proteínas Vegetales  
Universidad Nacional de La Plata  
La Plata 47 y 115, CC 711, 1900  
La Plata, Argentina  
Tel: 54 221 4235333 ext. 57  
Fax: 54 221 422 4067  
E-mail: [priolo@biol.unlp.edu.ar](mailto:priolo@biol.unlp.edu.ar)

**Financial support:** The present work was supported by grants from ANPCyT, CIC, CONICET, CYTED, and UNLP.

**Keywords:** *Asteraceae*, callus, rhizogenesis, *Silybum marianum*.

**Abbreviations:** 2,4-D: dichlorophenoxy acetic acid  
BA: benzyladenine  
NAA: naphthalenacetic acid

The objective of this work was the optimization of the conditions of *in vitro* culture for callus production of *Silybum marianum* (L.) Gaertn. (*Asteraceae*). Sections of cotyledons, previously disinfected by washing

successively with ethanol 70%, NaClO (10% w/v) and Tween 20 (0.05% v/v) and rinsing with sterile distilled water, were used as explants. For its initial culture, B5 medium supplemented with BA and 2,4-D solidified

\*Corresponding author

with phytigel was used, and a 63% survival was achieved. To obtain callus, two solid media were assayed (S1 and S2) using B5 medium supplemented with growth regulators (BA and 2,4-D or NAA and BA, respectively). The calli were grown at 25°C during 45 days in darkness. Growth kinetics was studied using S1 medium obtaining a typical growth curve with an exponential phase after 14 days of incubation (rate of growth 0.005 g dry weight/ day) and stationary phase after 35 days. The rate of growth in S2 medium was slower, and rhizogenesis was observed starting on the fifth week of incubation. From these results, the best culture medium for callus production of *Silybum marianum* was S1 medium.

Enzyme preparations, from extracts of plants or animal tissue, were used well before much was known about the nature and properties of enzymes. The great majority of commercial enzymes have been obtained mainly from microbial sources. Plant enzymes, such as papain, bromelain and ficin (cysteine peptidases) are employed in different industrial processes and medicine (Uhlig, 1998). All enzymes employed in milk coagulation are aspartic peptidases, with acidic optima pH, and possess high levels of homology between their primary structures and similarity between their catalytic mechanisms (Silva and Malcata, 1999).

The flowers of different species of cardoon (*Asteraceae*) have been characterized because they are a rich source of aspartic peptidases with milk clotting activity (Verissimo et al. 1996; Cordeiro et al. 1998; Llorente et al. 2004). Since Roman times, aqueous extracts of *Cynara cardunculus* flowers have been used as a milk coagulant in the manufacture of various types of the Iberian Peninsula traditional cheeses such as Serra da Estrela cheese, Serpa, Azeitão, Los Pedroches, La Serena and Flor de Guía (Silva and Malcata, 1999; Silva et al. 2002).

The presence of aspartic peptidases with milk clotting activity has been detected in crude aqueous extracts of flowers of milk thistle, *Silybum marianum* (L.) Gaertn. (*Asteraceae*), (Vairo Cavalli et al. 2005) and the use of its flowers and leaves for the production of small-scale Serpa cheese has been reported (Cabral et al. 1984).

One of the reasons for research on various plant cell, tissue or organ cultures is the ability of these cultures to synthesize *in vitro* some of the metabolites that are found in the whole plants (Tamer and Mavituna, 1996). Thus, this becomes an alternative for obtaining products that are difficult to obtain by conventional methods or whose production is not economically viable. Furthermore, plant enzyme supply tends to be erratic and influenced by climate and season which determinates its uselessness in a full-scale cheese industry.

Cabral et al. (1984) have immobilized cells and protoplasts obtained from *S. marianum* cell suspension cultures with

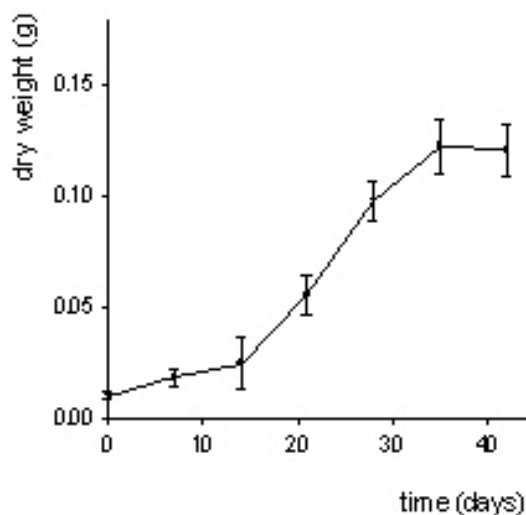


Figure 1. Variation of dry weight from *S. marianum* callus obtained in S1 medium during growth time.

milk clotting activity, but the peptidases responsible for the activity have not been characterized.

The aim of the present work is the optimization of callus culture of *Silybum marianum* (*Asteraceae*), milk thistle, for biomass production as a potential source of milk clotting peptidases. The media chosen were selected due to its usefulness for the *in vitro* production of peptidases with milk clotting activity in different species of *Asteraceae*. (Fevereiro et al. 1986).

## MATERIALS AND METHODS

### Explants

Achenes of *Silybum marianum* (L.) Gaertn. (Marzocca, 1957) were washed under tap water for 24 hrs and began to germinate in seedbeds for 2 weeks and kept at an 8 hrs light / 8 hrs dark photoperiod. The seedbeds remained during this time in trays with provision of water. The plantlets were scrupulously washed and its cotyledons were chopped in two parts that were used as explants for callus production.

### Plant material disinfection

For disinfection of plant material destined to *in vitro* cultures, assays were done varying the times of exposition and the concentrations of the agents used (ethanol and sodium hypochlorite). The method selected consisted in disinfecting the plant material successively washing it with ethanol 70° for 5 min, NaClO 10% for 10 min and Tween 20 (0.05% v/v) for 15 min. They were finally washed thoroughly three times with sterile distilled water in a laminar flux chamber.

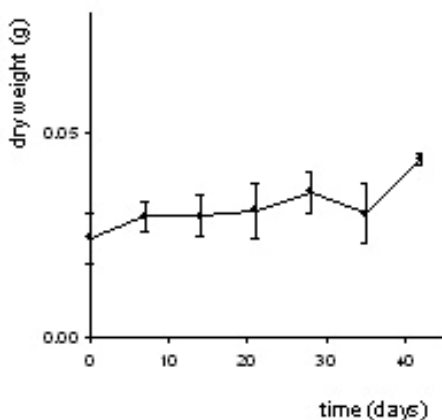


Figure 2. Variation of dry weight from *S. marianum* callus obtained in S2 medium during growth time

### Callus initiation and production

The basal medium used was B5 (Gamborg et al. 1968) supplemented with 0.05 mg/l of BA and 0.5 mg/l of 2,4-D, and solidified with 2.5 g/l of phytigel. The pH was adjusted to 5.8. Explants obtained from 60 plantlets were incubated at  $24 \pm 2^\circ\text{C}$  in darkness during 45 days.

The calli obtained this way were inoculated in two solid media. The media assayed (S1 and S2) were B5 medium solidified with 2.5 g/l of phytigel and supplemented with growth regulators (BA and 2,4-D or NAA and BA respectively). S1 medium was supplemented with 0.05 mg/l of BA and 0.5 mg/l of 2,4-D; S2 medium was supplemented with 0.025 mg/l of BA and 0,01 mg/l of NAA. These hormonal supplements were selected because they were optimum for the production *in vitro* of peptidases with milk clotting activity in different species of *Asteraceae* (Fevreiro et al. 1986). In both cases the pH was adjusted to 5.8. The media were sterilized in autoclave at 1 atmosphere of overpressure for 20 min. The callus were grown at  $24 \pm 2^\circ\text{C}$  during 45 days of incubation in the darkness.

Growth kinetics was studied by determination of dry weight of fresh callus at 7, 14, 21, 28, 35 and 42 days old. Dry weight of fresh callus was determined after drying in a vacuum oven at  $65^\circ\text{C}$  until constant weight.

### RESULTS AND DISCUSSION

After surface sterilization, 63% of survival was achieved and 25% of the samples were contaminated. Longer periods of contact with the NaClO produced the death of the explants. A good response was obtained when the explants were inoculated on the selected medium.

Other authors when assaying different media for the obtention of calli of *Cynara cardunculus* supplemented with different combinations of kinetin and NAA, combinations of kinetin and 2,4-D, and combinations of BA and 2,4-D, have obtained the best biomass production using B5 medium supplemented with BA and 2,4 D in relation 1:10 (Figueiredo et al. 1987). In the present work the best response in terms of biomass production was obtained using S1 medium.

Growth kinetics in S1 medium showed a typical curve with an exponential growth phase that began after 14 days of incubation and stationary growth phase that began after 35 days. The rate of growth during the exponential growth phase was of 0.005 g (dry weight)/day, with correlation coefficient of 0.99 (Figure 1).

On the contrary, the rate of growth in S2 medium was slower (Figure 2), and produced rhizogenesis (Figure 3) starting on the fifth week of incubation. When media supplemented with different proportions of BA and NAA for obtaining of calli from *Cynara scolymus* L. (*Asteraceae*) were assayed, the best results were achieved using a ratio BA/NAA of 1:10.

### CONCLUDING REMARKS

From the results obtained, the best culture medium for callus production for biomass production of milk thistle (*Silybum marianum*) as a potential source of milk clotting peptidases was B5 medium solidified with 2.5 g/l of phytigel and supplemented with 0.05 mg/l of BA and 0.5 mg/l of 2,4-D.

### ACKNOWLEDGMENTS

S. Vairo-Cavalli is awarded fellowship by CONICET and C.L. Natalucci is member of CIC Researcher Career. Authors also thank Tolbiac S.R.L. and Mr. José Madarnas for the supply of de *Silybum marianum* seeds.



Figure 3. Rhizogenesis from culture callus in S2 medium.

## REFERENCES

- CABRAL, J.M.S.; FEVEREIRO, P.; NOVAIS, J.M. and PAIS, M.S.S. Comparison of immobilization methods for plant cells and protoplasts. *Annals of the New York Academy of Science*, 1984, vol. 434, no. 2, p. 501-503.
- CORDEIRO, M.; LOWTHER, T.; DUNN, B.; GURUPRASAD, K.; BLUNDELL, T.; PAIS, M.S. and BRODELIUS, P.E. Substrate specificity and molecular modeling of aspartic proteinases (cyprosins) from flowers of *Cynara cardunculus* subsp. *flavescens* cv. cardoon. In: JAMES, M. ed. *Aspartic Proteinases*. New York: Plenum Press, 1998, p. 473-479.
- FEVEREIRO, P.; CABRAL, J.M.S.; FONSECA, M.M.R.; NOVAIS, J.M. and PAIS, M.S.S. Callus and suspension culture of *Silybum marianum*. Biosynthesis of proteins with clotting activity. *Biotechnology Letters*, 1986, vol. 8, no. 1, p. 19-24.
- FIGUEREIDO, A.C.; FEVEREIRO, P.; CABRAL, J.M.S.; NOVAIS, J.M. and PAIS, M.S.S. Callus and suspension culture for biomass production of *Cynara cardunculus* (*Compositae*). *Biotechnology Letters*, 1987, vol. 9, no. 3, p. 213-218.
- GAMBORG, O.L.; MILLER, R.A. and OJIMA, K. Nutrient requirements of suspension culture of soybean root cells. *Experimental Cell Research*, 1968, vol. 50, no. 1, p. 151-158.
- LLORENTE, B.; BRUTTI, C. and CAFFINI, N.O. Purification and characterization of a milk-clotting aspartic proteinases from globe artichoke (L). *Journal of Agriculture and Food Chemistry*, 2004, vol. 52, no. 26, p. 8182-8189.
- MARZOCCA, A. *Silybum marianum* (L.) Gaertn. In: *Manual de Malezas*. Buenos Aires: Imprenta y Casa Editora Coni, 1957, p. 435-437.
- SILVA, S.V. and MALCATA, F.X. On the activity and specificity of cardosin B, a plant proteinase, on ovine caseins. *Food Chemistry*, 1999, vol. 67, no. 4, p. 373-378.
- SILVA, S.V.; BARROS, R.M. and MALCATA, F.X. Hydrolysis of caseins by extracts of *Cynara cardunculus* precipitated by ammonium sulfate. *Journal of Food Science*, 2002, vol. 67, no. 5, p. 1746-1751.
- TAMER, I. Melih and MAVITUNA, Ferda. Protease from callus and cell suspension cultures of *Onopordum turcicum*. *Biotechnology Letters*, April 1996, vol. 18, no. 4, p. 361-366.
- UHLIG, Helmut. *Industrial enzymes and their applications*. Willey & Sons, Nueva York, 1998. p. 472. ISBN: 0-471-19660-6.
- VAIRO CAVALLI, Sandra; CLAVER, Santiago; PRIOLO, Nora and NATALUCCI, Claudia. Extraction and partial characterization of a coagulant preparation from *Silybum marianum* flowers. Its action on bovine caseinate. *Journal of Dairy Research*, 2005, vol. 72, no. 3, p. 271-275.
- VERÍSSIMO, P.; FARO, C.; MOIR, A.; LIN, Y.; TANG, J. and PIRES, E. Purification, characterization and partial amino acid sequencing of two novel aspartic proteinases from the flowers of *Cynara cardunculus* L. *European Journal of Biochemistry*, 1996, vol. 235, no. 3, p. 762-768.