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RESEARCH ARTICLE

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

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Abbreviations: 2,4-D: dichlorophenoxy acetic acid
BA: benzyladenine
NAA: naphtalenacetic 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 70º, 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

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with phytagel 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 25ºC 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 (Veríssimo 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 milk clotting activity, but the peptidases responsible for the activity have not been characterized.

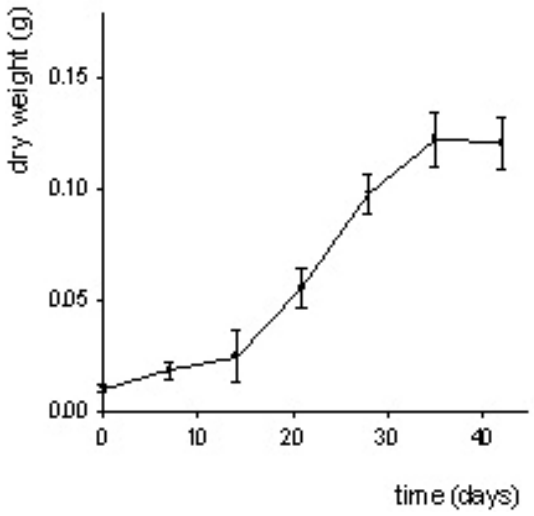

Figure 1. Variation of dry weight from *S. marianum* callus obtained in S1 medium during growth time.

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^{o} 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.

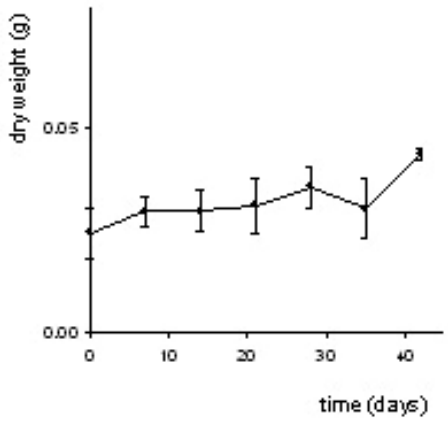

Figure 2. Variation of dry weight from *S. marianum* callus obtained in S2 medium during growth time

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 phytagel. The pH was adjusted to 5.8. Explants obtained from 60 plantlets were incubated at 24 ± 2ºC 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 phytagel 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* (Fevereiro 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 ± 2ºC 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 65ºC 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 phytagel and supplemented with 0.05 mg/l of BA and 0.5 mg/l of 2,4-D.

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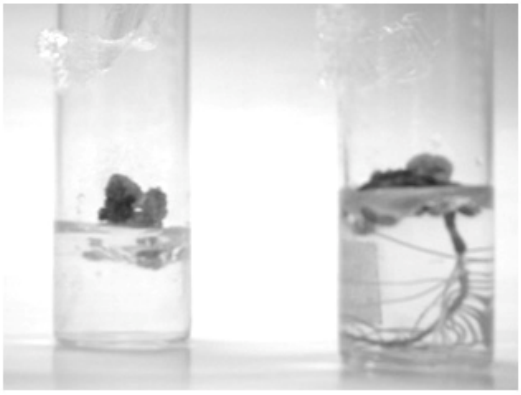

Figure 3. Rhizogenesis from culture callus in S2 medium.

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