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Effect of plant extracts on development of *Capsella* embryos in ovules cultured *in vitro*

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Abstract

When distantly related plants are crossed, embryos abort on the mother plant. One of the methods used to rescue the embryo is to cultivate the ovule *in vitro*. When the ovule is precociously detached from the mother plant, survival rate of the embryo is very low. To increase the chance of survival as well as the growth of the embryo, the medium was supplemented by different extracts of plants after either autoclaving or filtering. Autoclaved tomato extract seemed to have no simulating effect in spite of reports from different authors. Filtered coconut milk increased growth and filtered cucumber extract improved survival. Other new extracts had a stimulating action on both growth and survival and are of interest for further study.

Introduction

When distantly related plants are crossed, hybrid embryos usually stop growing and degenerate. Only early transfer of embryos or ovules to an artificial medium can ensure their development into plants.

One useful method which allows development of smaller embryos is to cultivate the whole ovule instead of the isolated embryo (Monnier 1984). In this way embryos of 25 μm can develop inside the ovules with a survival of 40 % (Lagriffol and Monnier 1985, Monnier and Lagriffol 1986). Nevertheless, when the embryo is 12 μm long the survival falls to 15 % (Lagriffol and Monnier 1983) and although numerous media have been tested (Monnier 1984) no increase in the survival rate occurred. Probably, *in situ*, some unknown factors are brought by plant to allow the very early development of the embryo inside the ovule. One approach to try to increase the percentage of survival is to test the effect of crude plant extracts. These extracts are made with organs which store food in plants (fruit, endosperm, cotyledon). It has been known, for a long time, that plant extracts improved the development of embryos in culture.

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Van Overbeek *et al.* (1944) showed that addition of coconut milk stimulated the growth of young *Datura* embryos *in vitro* and Norstog (1961) confirmed this observation with barley embryos. Vacin and Went (1949) and Nitsch (1954) used tomato juice to improve the development of orchid embryos and *Helianthus* tissue, respectively. Nakajima *et al.* (1969) employed watermelon and cucumber juice in the culture of young ovules of *Trifolium repens* and demonstrated that when the extracts were filtered they had a promotive effect on the growth of the embryos. Later Wakizuka and Nakajima (1975) confirmed this action on *Petunia* embryos enclosed in ovules. More recently it has been reported that extracts of incubated cotyledons could notably increase the growth of embryonic axes of *Phaseolus* (Monnier 1982).

All these experiments were made on embryos of different species. In this study the effects of different plant extracts were tested on a single species *(Capsella bursa-pastoris)* in order to obtain useful information on their relative effectiveness. In addition, although these extracts were reported to increase growth rates of embryos, there was no information on their effect on survival of embryos. This information is important as, in case of crossing, it is necessary to culture the *in ovulo* embryo as early as possible (*i.e.* before abortion) to rescue a maximum number of hybrid embryos. Besides, a growth-promoting medium can be inadapted to culture small embryos since growth and survival may respond in oposite ways (Monnier 1976).

This study was made to determine what are the most efficient extracts before analysis and identification of the growth and survival-promoting factors.

Material and methods

Plant material: The plant employed was *Capsella bursa-pastoris* of the cruciferous family. This plant is very useful because its embryogenesis has been well studied (Schaffner 1906; Soueges 1919). Plants were cultivated in a growth chamber at 18 °C with continuous lighting.

Culture basal medium: Culture conditions have been reported previously (Lagriffol and Monnier 1985). The medium was composed of a mineral solution (Monnier 1976), evolved from Murashige and Skoog's (1962) to meet the special requirements of immature *Capsella* embryos. This solution was supplemented with 400 mg l^{-1} of glutamine, 80 g l^{-1} of sucrose (this quantity is the optimal concentration for the culture of ovules), 1 mg l^{-1} of each vitamin B1 and B6 and 7 g l^{-1} of Difco agar. This medium was called the basal medium and prepared at double concentration. Thereafter the extract was added to the basal medium and the whole mixture was adjusted to 1 l with distilled water.

The extract concentrations ranged between two limits: the lower concentration was chosen when an effect on growth was detectable, the higher concentration when survival begins to decrease.

Preparation of extracts of plants: Fruits of tomatoes (*Lycopersicum esculentum* cv. Dona) and cucumber (*Cucumis sativus* cv. Fl Admirable) were obtained locally and

peeled before use. Immature coconuts *(Cocos nucifera)* from Africa were opened and the coconut milk was collected. Cotyledons of beans (*Phaseolus vulgaris* cv. Early dwarf white coco) were cultivated 12 d on agar medium before using them (Monnier 1982).

To ensure dissolving of the components of the plant organ, a certain quantity of distilled water, equal in weight to that of the organ, was added before grinding in a mixer. The mixture was centrifuged (100 rev. s^{-1} for 30 min). The supernatant was collected and its pH was adjusted to 5.5 with NaOH. This liquid, called the extract, was added to the basal medium in different proportions. The extract was sterilized either by heat (autoclave: 110 °C, 10 min) or by ultrafiltration (filtration on Büchner funnel then on *Millipore* filter: prefilter and 0.47 µm filter). In that way thermolabile substances were not destroyed.

After sterilisation this extract was incorporated to the basal medium in the liquid phase, prior to solidification, and the mixture was poured into sterilized glass Petri dishes.

Inoculation of ovules: *Capsella* fruits were opened under a dissecting microscope, the ovules detached and cultured singly on the medium as described earlier (Lagriffol and Monnier 1983).

Ovules according to their size contain embryos of different length. 500 µm long ovules were chosen because they contain embryos whose average length is 25 µm. 25 µm long embryos were chosen because their survival was close to 50 %. This value, which varied greatly when toxicity began to appear as the concentration of the extract increased, could therefore perfectly inform us about the suitability of the extract.

Cultures were incubated at 25 °C in darkness for 6 d. This time corresponds to the end of the logarithmic growth of the embryo and also of the ovule (Lagriffol and Monnier 1983).

Survival and growth evaluation:. After 6 d of culture the ovule was dissected and the length of the embryo was measured by an ocular micrometer. This measurement was accurate to ± 6 µm. Growth percentage was obtained by calculating the ratio:

$$\frac{L_6 - L_0}{L_0} \times 100$$

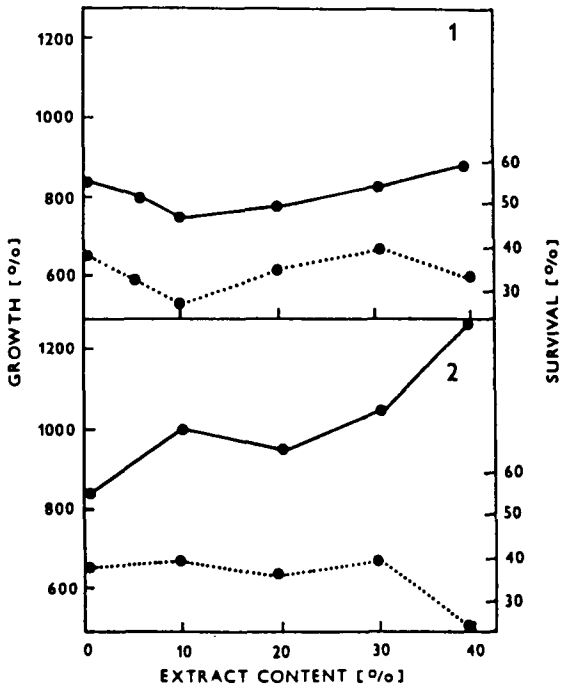
where L_0 was the length of the embryo at the time of inoculation (25 µm) and L_6 the length of the embro after a 6-d culture period. Growth curves only contain the data concerning the surviving individuals.

The percentage of survival was expressed as the ratio of the number of live embryos and the number of transplanted embryos multiplied by 100. An embryo was considered alive if, after 6 d of culture, it showed an increase in length of at least 25 µm. A previous histological study had showed us that if the embryo length doubled, this indicated that there was not only an enlargement of cells but a multiplication of cells.

For statistical analysis the comparison betwen two percentages was based on the reduced deviation ε for a risk of 5 %. If ε was superior to 1.96 the difference between the two percentages was considered to be significant. Each point of the curves represents the mean of measurements of 120 ovules inoculated.

Results

Effect of coconut milk: The curve of the growth percentage shows little variation with concentration of the autoclaved coconut milk (Fig. 1). In addition there is no significant difference to the control. The variation in the rate of survival is also negligible.

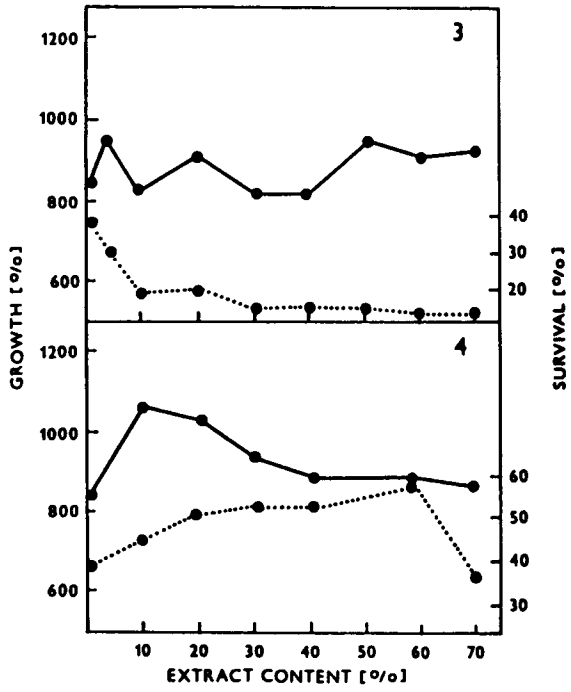


Figs. 1 and 2.. Effects of autoclaved (Fig. 1) or filter-sterilized (Fig. 2) coconut milk on growth (*full line*) and survival (*dotted line*) of *Capsella* embryos.

Within the range of concentration tested, autoclaved coconut milk has no significant effect on either growth or survival. Fig. 2 shows an increase in the rate of growth with increasing concentrations of filter-sterilized coconut milk. Growth is significantly different from the control (ε = 2.8) from a concentration of 30 %.

Survival is constant at all concentrations up to 30 %, thereafter a sharp decrease occurred which was significantly different from the control (ε = 2.4).

Effect of cucumber extract: Autoclaved cucumber extract has no significant effect on growth (Fig. 3). The percentage of survival however decreased steadily when the concentration increased. The autoclaved extract of cucumber has a toxic effect on survival of young embryos.



Figs. 3 and 4. Effects of autoclaved (Fig. 3) or filter-sterilized (Fig. 4) cucumber extracts on growth (*full line*) and survival (*dotted line*) of *Capsella* embryos.

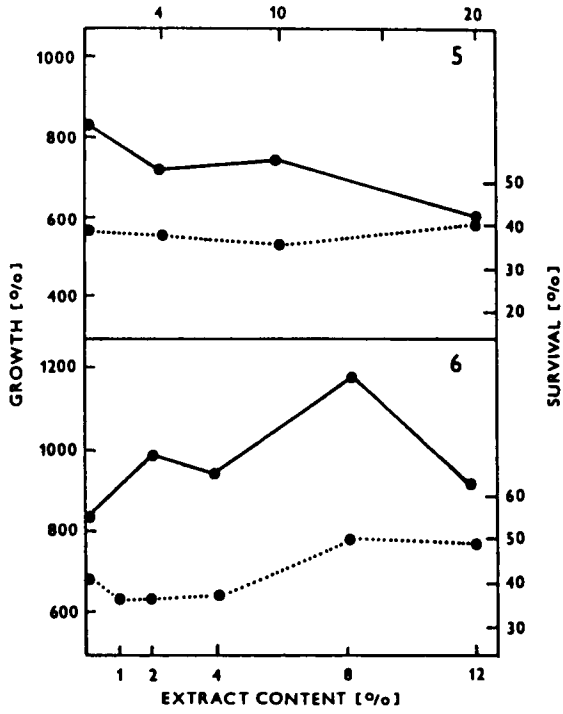
Fig. 4 shows a significant increase in the percentage of growth for the concentration of 10 % of filter-sterilized cucumber extract (ε=2.61). This stimulating effect diminishes progressively without any toxic effect on survival for the concentration tested to 60 %.

Filtered extract of cucumber was therefore beneficial for both growth and survival of embryos but not at the same concentration.

Effect of tomato extract: Since it was recommended that tomato extract was autoclaved (Vacin and Went 1949, Nitsch 1954) no experiments was performed with filtered extract.

When the concentration of autoclaved tomato extract increased there was a constant decline in the percentage of growth (Fig. 5). At a concentration of 20 % of

tomato extract, the growth was significantly inhibited ($\varepsilon = 2.46$), however not yet the percentage of survival.



Figs. 5 and 6. Effects of autoclaved tomato (Fig. 5) or bean (Fig. 6) extracts on growth (*full line*) and survival (*dotted line*) of *Capsella* embryos.

Effect of autoclaved bean extract: The extract was made from cotyledons incubated 12 d on water-agar (Monnier 1982). The extract was difficult to filter and, because some preliminary experiments had shown that a filtered extract was generally not very beneficial to the embryos, we only tested the autoclaved extract. With 2 % extract there was an increase of growth rate (Fig. 6) which was already significantly different from the control (ε=2.09). The optimal concentration for both growth and survival was 8 %.

Discussion

Regarding the growth of embryos the results obtained, were in general identical to those reported by the authors earlier. The difference between autoclaved and filter-sterilized coconut milk had already been observed by Van Overbeek *et al.* (1944). As it was confirmed, coconut milk contains thermolabile substances which increase the

growth of embryos from a concentration of 30 % to 40 % and which are destroyed by heat during sterilization. In the same way it is only the filtered cucumber extract which was beneficial to the embryos, this effect disappeared when it was autoclaved. This extract as coconut milk contain thermolabile substances. Indeed when cucumber extract was tested by Nakajima *et al.* (1969), and Wakizuka and Nakajima (1975), only a filtered extract was used. Concerning bean extract, as it was reported (Monnier 1982), it has a stimulating effect, when autoclaved, on embryonic axes of *Phaseolus*, specially at the concentration of 8 %.

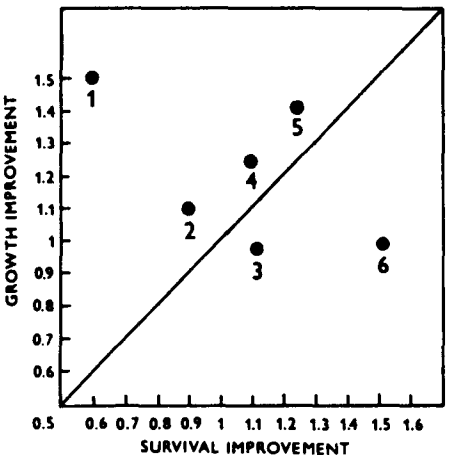


Fig. 7. Improvement of growth and survival of *Capsella* embryos by addition of extracts.
1 - filtered coconut milk (40 %); 2 - autoclaved coconut milk (40 %); 3 - autoclaved coconut milk (30 %); 4 - filtered cucumber extract or coconut milk (10 %); 5 - autoclaved bean cotyledon extract (8 %); 6 - filtered cucumber extract (60 %)

But, on the contrary, with some extracts the observations were different from the authors. For instance with tomato extract there was no improvement of growth. At the concentration of 20 % no change of the survival rate could be observed but there was a significant decrease of growth. In conclusion it appears that tomato extract contains substances which reduce growth but not survival. This result differed from the observations of Vacin and Went (1949) and Nitsch (1954) possibly because of the use of a different type of cultivated material.

In addition to the authors, we have considered the survival of embryos. In order to compare the best results, the ratios:

$$\frac{\text{optimal growth with extract}}{\text{growth of control}} \quad \text{and} \quad \frac{\text{optimal survival with extract}}{\text{survival of control}}$$

were calculated and respectively called growth and survival improvement. Only the stimulating effects of the extract, *i.e.* when one at least of the ratios was superior to 1, were plotted (Fig. 7). In Fig. 7, the inferior point of each label marks the survival and growth improvement. Some extracts like coconut milk at the concentration of

40 % increased growth of embryos but survival was reduced. On the contrary, the filtered cucumber extract for the concentration of 60 % improved the survival but had no effect on growth. Other extracts had a stimulating effect on both growth and survival. The best extract which provided at the same time an improvement of survival and growth was the autoclaved cotyledon extract.

This research allowed us to choose the best extract (autoclaved cotyledons) and this extract will be analyzed in order to determine what kind of elements was able to promote, at the same time, growth and survival of embryos.

The growth promoting factors in this extract are probably not the mineral salts because the mineral solution was carefully adjusted to grow immature *Capsella* embryos (Monnier 1976). A certain number of vitamins have also been tried without any success. These growth promoting substances could be plant growth regulators like cytokinins as it was discovered in extracts of *Lupinus* seeds (Matsubara and Koshimizu 1966).

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