PRODUCTION OF EMBRYOS FROM MICROSPORE CULTURES OF PORTUGUESE TRONCHUDA CABBAGE LANDRACES

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Abstract

Twelve accessions of tronchuda cabbage landraces were tested for their ability to produce embryos through microspore culture in NLN-13 medium. A sample from all the isolations was stained with DAPI for determination of microspore developmental stage. The relationship between the microspore developmental stage and the production of microspore-derived embryos was evaluated. Embryos were obtained from all the accessions. Considerable variation was observed between isolations with different developmental stage of microspores, accessions and plants within the same accession. The best embryogenic responses occurred in microspore populations with approximately 10 to 89% of binucleate pollen. The highest embryo yield was obtained with one of the 'Couve de Valhascos' accessions (0.41 embryos / 1 000 microspores) and the lowest yields with the accessions of 'Couve Portuguesa' (less than 0.07 embryos / 1 000 microspores).

Keywords: *Brassica oleracea* var. *costata*, Portuguese coles, microspore culture, embryogenesis, microspore stage, genetic variation

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1. Introduction

Landraces of tronchuda cabbage (*Brassica oleracea* var. *costata* DC. syn. var. *tronchuda* Bailey) are a unique and diversified group of vegetables very important in the Portuguese diet. The extensive use and long history of cultivation of these coles in Portugal gave rise to a tremendous diversity. There are different ecotypes all over the country; these differ in ecological adaptation, vegetative cycle and several morphological characters (Dias, 1995).

Recently a tronchuda cabbage breeding programme was initiated at Instituto Superior de Agronomia for the development of open pollinated cultivars and F_1 hybrids. Microspore culture is an effective technology that can decrease the time required to produce homozygous lines (replacing self-pollination) for the production of F_1 hybrids and it increases the selection efficiency for desirable genetic recombinants.

The first report of successful microspore culture in *Brassica* was made by Lichter (1982) in rapeseed (*B. napus*). Improvements in the efficiency of microspore embryogenesis in rapeseed have been achieved in recent years and many factors such as microspore developmental stage, genotype, culture medium, method of microspore isolation and culture conditions have been studied (Swanson et al., 1987; Chuong et al., 1988; Gland et al., 1988; Kott et al., 1988).

The application of this technique to other *Brassica* species has been more limited but successful microspore culture has been reported for *B. campestris* (Ohkawa et al., 1988; Lichter, 1989; Burnett et al., 1992; Baillie et al., 1992), *B. carinata* (Chuong & Beversdorf, 1985), *B. nigra* (Lichter, 1989; Hertz & Schieder, 1991; Margale & Chèvre, 1991) and *B. juncea* (Ohkawa et al., 1988). In *B. oleracea*, there are reported studies for several crop types: broccoli (var. *italica*) (Lichter, 1989; Takahata & Keller, 1991; Duijs et al., 1992), kailan (var. *alboglabra*) (Takahata & Keller, 1991), cabbage (var. *capitata*) (Lichter, 1989; Cao et al., 1990; Duijs et al., 1992), cauliflower (var. *botrytis*), curly kale (var. *fimbriata*), savoy cabbage (var. *sabauda*) and Brussels sprouts (var. *gemmifera*) (Duijs et al., 1992). Studies on microspore culture in *B. oleracea* var. *costata* have thus far not been reported.

The objective of the present study was to investigate the ability of tronchuda cabbage landraces to produce embryos through microspore culture. Special attention was given to the developmental stage of the microspores and to accession and plant effects.

2. Materials and methods

Plant genotype. Twelve accessions of Portuguese tronchuda cabbages were used in the present study. Accession designations and their sources are listed in Table 1. The accessions were selections obtained at the Instituto Superior de Agronomia from commercial seeds or from seeds collected directly from the growers in different regions of Portugal.

Plant growth conditions. Plants were grown in the open air in plastic pots of 16 cm diameter filled with clay soil - Levington M26 (1:1) and watered and fertilized as required. Then they were vernalized in a cold room at 4+1°C with continuous light of 10 μEm-2s-1 for four to eight

weeks according to the accession requirements. For generative development plants were transferred to a culture room at 22±1°C with continuous light of 50 μEm-2s-1.

Isolation of microspores. For both isolation and culture of the microspores, a filter sterilized Nitsch & Nitsch medium (1967) as modified by Lichter (1981) with 13% sucrose (NLN-13), without potato extract or growth regulators, at pH 6.1, was used. Microspore culture was performed three to five times using three to four plants of each accession. Depending on the number of young inflorescences, six to ten groups of two to four flower buds with the same length harvested from single plants, were made. The buds were sterilised in a 1.7% (w/v) solution of sodium dicloroisocyanurate with a few drops of Nonidet P-40® for 6 min and washed three times in sterile water (6 min each time). Each group of buds was macerated in a beaker containing 1.5 ml of NLN-13 medium. A microspore suspension was obtained by filtration through two 45 um nylon screens. This suspension was centrifuged 3 times at 900 rpm for 3 min and resuspended at a density of 40 000 microspores/ml in the same medium. Two millilitres of the microspore suspension was incubated in a 35x10 mm plastic Petri dish. Five to ten dishes were made per single plant isolation. For each isolation one hundred microspores stained with the DNA specific fluorochrome DAPI (4',6-diamidino-2-phenylindole) were always observed and scored according to nuclear morphology as early uninucleate, mid uninucleate, late uninucleate microspores and binucleate or trinucleate pollen (Kott et al., 1988; Telmer et al., 1992).

Culture of microspores. Microspores were incubated in darkness at 30±0.1°C for 48 h and then maintained at 25±0.1 °C in darkness. After three weeks, the cultures were transferred to a gyratory shaker and agitated at 60 rpm in the dark at 24±0.1°C. The embryo number was scored four weeks after microspore isolation.

Data analysis. Results were expressed as number of embryos per Petri dish (approximately 80,000 microspores).

Based on the cytological data of the microspores in each isolation; eight classes of microspore development were established for all accessions studied according to the percentage of uninucleate microspores. The composition of these classes is as follows:

A. 100% uninucleate microspores, with less than 60% late uninucleate

B. 100% uninucleate microspores, with more than 60% late uninucleate

C. 90-99% uninucleate microspores (1-9% binucleate pollen)

D. 70-89% uninucleate microspores (10-29% binucleate pollen)

E. 40-69% uninucleate microspores (I30-59% binucleate pollen)

F. 10-39% uninucleate microspores (l60-89% binucleate pollen)

G. 1-9% uninucleate microspores (190-99% binucleate pollen)

H. 0% uninucleate microspores (100% binucleate and trinucleate pollen)

It should be pointed out that classes E, F and G do not always correspond exactly to classes with 30 to 59%, 60 to 89% and 90 to 99% of binucleate microspores respectively due to the presence of trinucleate and binucleate pollen as well as uninucleate microspores in some isolations of these classes.

The analysis was performed using Generalised Linear Models with the programme Genstat 5 assuming that the data follows the Poisson distribution. The means were compared according to pairwise t tests. The interaction class' accession could not be fully included in the model because some accessions did not have data in all the classes. Separated analyses were made for the term class and for the term accession. The classes were compared in all the data and then within each accession and the accessions were compared in all the data and then within each class.

3. Results

3. 1. Microspore developmental stage

Bud length was not well related to the developmental stage of microspores (Vicente, 1995).

The overall effect of the term microspore developmental class was highly significant ($P<0.001$) (Vicente, 1995). Table 2 presents the embryo production in successive microspore development classes. Class E (40 to 69% of uninucleate microspores) was the most productive with 6.19 embryos per dish followed by classes D (70 to 89% of uninucleate microspores) and F (10 to 39% of uninucleate microspores) with 5.22 and 5.19 embryos per dish respectively. Younger and older classes (A, B, C and G, H respectively) were less productive.

For individual accessions, the effect of the term microspore developmental class was also highly significant ($P<0.001$) with exception of accessions 5 and 6 in which it was not significant (Vicente, 1995).

Figure 1 shows for each accession the mean production of embryos in the various microspore developmental classes. The highest yields in each accession were not always in the same class of developmental stage of microspores. The most productive classes per accession were: class D in 'Penca de Mirandela' (1), 'Penca da Póvoa' (2) and 'Couve Portuguesa' (4); class E in 'Couve Glória de Portugal' (3) and 'Couve de Valhascos' (10); class F in 'Couve Portuguesa' (7), 'Couve de Valhascos' (9), 'Couve Murciana' (11) and 'Couve Algarvia' (12); class G in 'Couve Grelo' (8).

3.2. Accession effect

The overall effect of the term accession was highly significant ($P<0.001$) and it explained most part of variation (Vicente, 1995). Table 3 shows the embryo production for the various

accessions. 'Couve de Valhascos' (10) was the most productive accession with 12.36 embryos per dish followed by 'Couve Grelo' (8) with 6.90 embryos per dish. All the other accessions had relatively lower yields (with less than 4.0 embryos per dish). The accessions of 'Couve Portuguesa' (4, 5, 6, 7) were the less productive (0.08 to 0.53 embryos per dish) followed by 'Couve Murciana' (11) (1.03 embryos per dish). The yields of the two accessions of 'Couve de Valhascos' (9 and 10) were significantly different.

In class H (0% of uninucleate microspores) the effect of the term accession was not significant but in all other classes it was highly significant ($P<0.001$) (Vicente, 1995). The most productive accessions per class were (Figure 1): 'Couve de Valhascos' (10) in classes A, D, E and F, 'Penca de Mirandela' (l) in class B: 'Penca de Mirandela' (l) and 'Couve de Valhascos' (10) in class C; 'Couve Grelo' (8) in class G.

3.3. Plant effect

A considerable variation among embryo yields in different plants of the same accession was also observed. For example, in 'Couve Grelo' (8), three plants generated yields of more than 12 embryos per dish and one plant yielded an average of less than one embryo per dish (data not shown).

4. Discussion

Microspore embryogenesis was demonstrated in all the tronchuda cabbage accessions tested.

In tronchuda cabbage a balanced proportion of late uninucleate microspores and binucleate pollen seems to be important for high embryo yields since the best embryogenic responses were among populations with 90 to 11% of uninucleate microspores or aproximately 10 to 89% of binucleate pollen (classes D, E and F). The presence of uninucleate microspores appeared to be necessary for embryogenesis since only two embryos were formed in class H (Table 2).

These facts are in agreement with the results obtained in *B. napus* cv. Topas by Telmer et al. (1992) which indicate that the best embryogenic responses were in populations with 1 to 87% of binucleate pollen and with the studies in the same cultivar of Fan et al. (1988) and Pechan & Keller (1988) which indicate that embryos are mainly obtained from microspores in the late uninucleate and from pollen in the early binucleate stage. However, our results contrast with those obtained in *B. napus* by Kott et al. (1988) which indicate that the best productions occur in populations in the late uninucleate stage just prior to the first mitosis.

The range of optimal developmental stages was fully in accordance with the range reported by Duijs et al. (1992) in several varieties and cultivars of *B. oleracea* (10 to 40% binucleate pollen) and by Cao et al. (1990) in *B. oleracea* var. *capitata* (40 to 50% binucleate and trinucleate pollen).

Class C produced fewer embryos than class B. This fact may be due to presence in class C of

a higher proportion of dishes of accessions with poor embryogenic responses than in class B.

The embryo production in tronchuda cabbage was highly influenced by the accession. This fact is also reported for cultivars of *B. napus* (Chuong et al., 1988; Kottet al., 1988; Siebel & Pauls, 1989), *B. campestris* (Baillie et al., 1992; Burnett et al., 1992) and *B. oleracea* (Cao et al., 1990; Takahata & Keller, 1991; Duijs et al., 1992).

The best embryo production of 33 embryos per dish (0.41 embryos/1000 microspores; 82 embryos / bud or 14 embryos / anther) was obtained from 'Couve de Valhascos' (10) in class E (Figure 1). This yield is lower than the best results reported in *B. napus* (Kott et al., 1988; Pechan & Keller, 1988) and *B. carinata* (Chuong & Beversdorf, 1985), but higher than the yields reported in *B. campestris* (Ohkawa et al., 1988; Litcher, 1989; Baillie et al., 1992; Burnett et al., 1992), *B. nigra* (Lichter, 1989; Hertz & Schieder, 1991; Margale & Chevre, 1991) and *B. juncea* (Ohkawa et al., 1988). It is also higher than yields obtained in *B. oleracea* by Lichter (1989) in cabbage and broccoli and by Cao et al. (1990) in several genotypes of cabbage, but is 10 times less than the yield obtained by Takahata & Keller (1991) in broccoli cv. Green Valiant and slightly inferior to the embryo yield of broccoli cv. New River reported by Duijs et al. (1992).

Martins (1992) performed anther culture in some accessions of Portuguese tronchuda cabbage. One accession of 'Couve Grelo' used in his study had the best yield (37 embryos per 100 anthers). The accession of 'Couve de Valhascos' was not reactive in contrast with our results. Microspore culture seems so to be a good alternative to anther culture in tronchuda cabbage. Siebel & Pauls (1989) have also reported that microspore culture was more efficient than anther culture in *B. napus*.

The fact that 'Couve de Valhascos' (10) and 'Penca de Mirandela' (1) appeared to have good embryo productions in early developmental stages (classes A and B respectively; cf. Figure 1) and 'Couve Grelo' (8) in late stages (class G) may be due to variability between plants since only one plant of each of these accessions had these responses. The differences between yields of different plants of the same accession may be due to genetic variation in the plant material since the accessions used in this study are heterozygous landraces with less than three years of selection.

This first study on microspore culture in tronchuda cabbages showed that this technology for production of haploid and double haploid homozygous plants is very amenable to these typical Portuguese coles. Embryo production varied between accessions tested, but in all accessions yields were high enough for application in breeding programmes. Some of the embryos produced were regenerated and the plants have been incorporated in our tronchuda cabbage genetic improvement programme.

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Table 1. Tronchuda cabbage accessions and their sources.

Accession number	Identification	Origin	Code[1]
1	Penca de Mirandela	Mirandela, Mirandela	ISA 453
2	Penca da Póvoa	Apúlia, Esposende	ISA 454
3	Couve Glória de Portugal	Arcozelo da Serra, Gouveia	ISA 84
4	Couve Portuguesa	Loures, Loures	CT-18/92
5	Couve Portuguesa	Loures, Loures	CT-20/92
6	Couve Portuguesa	Queluz, Sintra	CT-21/92
7	Couve Portuguesa	Loures, Loures	CT-29/92
8	Couve Grelo	Tojalinho, Loures	ISA 55
9	Couve de Valhascos	Valhascos, Sardoal	ISA36
10	Couve de Valhascos	Valhascos, Sardoal	CT42/92
11	Couve Murciana	Viana do Alentejo, Viana do Alentejo	CT-62/92
12	Couve Algarvia	Verdemilho, Aveiro	ISA 207

[1]ISA = Instituto Superior de Agronomia germplasm bank; CT = Selections of Tronchuda cabbages

Table 2. Embryo production in successive microspore developmental classes.

Class of microspores[1]	Number of dishes[2]	Mean (embryos / dish)
A	366	0.28 f
B	275	1.29 d
C	309	0.86 e
D	258	5.22 b
E	211	6.19 a
F	293	5.19 b
G	206	3.94 c
H	103	0.02 g

The means followed by thc same lower-case letter do not significantly differ at P=0.05 (according to pairwise (tests).

[1]Classes described at §2.

[2]Total number of Petri dishes incubated.

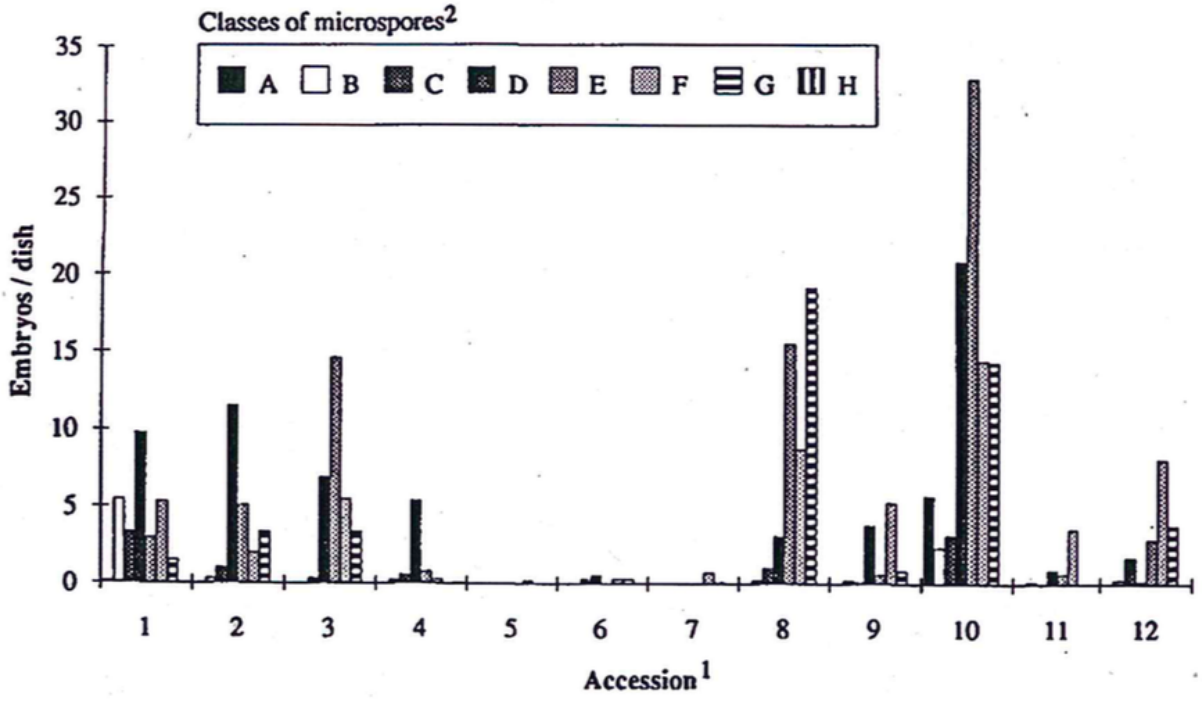

Figure 1. Mean production of embryos in various microspore developmental classes for twelve accessions of tronchuda cabbage.
[1]Accessions described in Table 1.
[2]Classes described at § 2.

Table 3. Embryo production in individual accessions of tronchuda cabbage.

Accession[1]		Number of dishes[2]	Mean (embryos / dish)
1.	Penca de Mirandela	168	3.96 c
2.	Penca da Póvoa	345	2.62 d
3.	Couve Glória de Portugal	138	2.51 d
4.	Couve Portuguesa	118	0.53 g
5.	Couve Portuguesa	77	0.08 i
6.	Couve Portuguesa	95	0.23 h
7.	Couve Portuguesa	257	0.10 i
8.	Couve Grelo	237	6.90 b
9.	Couve de Valhascos	138	1.84 e
10.	Couve de Valhascos	106	12.36 a
11.	Couve Murciana	182	1.03 f
12.	Couve Algarvia	160	1.83 e

The means followed by the same lower-case letter do not significantly differ at P=0.05 (according to pairwise t tests).
[1]Accessions described in Table l.
[2]Total number of Petri dishes incubated.