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Immature embryo rescue of grapevine (*Vitis vinifera* L) after an extended period of seed trace culture

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Summary

In this work the effect of time on *in vitro* culture of seed traces is evaluated. Without changing media, there was no decline in the number of rescued embryos for any cross within and up to 210 d of culture. After that period a decline was shown for varieties producing abundant callus from the external tegument. Direct germination of embryos increased in all crosses except for Blush Seedless x 73049, which stopped direct germination after 90 d. The polyembryony was advantageous for the cross Superior Seedless x Dawn Seedless, because it increased the percentage of normal plantlets. Multiple embryos showed a higher capacity to germinate in all cases. More embryos were obtained by excision and they presented a higher percentage of normal plantlets in comparison to plantlets obtained by embryos germinated directly. Embryos from the varieties Ruby Seedless, Blush Seedless and Bronx Seedless mainly produced normal plantlets while Crimson Seedless, Superior Seedless and Fantasy Seedless embryos mostly produced abnormal plantlets.

K e y w o r d s : *Vitis vinifera*, seedless grapes, embryo rescue.

Introduction

Seedless grapes which are preferred by consumers and thus by the raisin industry are classified in two types: stenospermocarpics and parthenocarpics. In the first type, the development of embryos and endosperms stops soon after fertilization and abortion of developing seeds occurs (Stout 1936). Seedless varieties have been obtained for more than 100 years (Tillery 1875) by emasculated seeded varieties pollinated with stenospermocarpic pollen. However, amateur breeders and gardeners have used the ancient seedless clones for centuries to cross them with favored seeded types (Ledbetter and Ramming 1989). Parthenocarpic varieties used as pollinators were not successful in the transmission of the seedless character (Snyder 1934).

Attempts have been made to obtain normal seeds of seedless varieties by the addition of plant regulators (Kender and Remaily 1970; Ledbetter and Shonnard 1990). This technique has not given positive results (Gargiulo 1991). In the past decades, embryo rescue techniques have been successfully applied in grapevine to obtain new seedless varieties (Cain *et al.* 1983; Emershad and Ramming 1984; Spiegel-Roy *et al.* 1985). The embryo rescue technique consists in culturing stenospermic seeds for several weeks, after which embryos are excised or plantlets (directly germinated embryos) are transplanted to a fresh developing medium. After some time, normal plants are hardened under greenhouse conditions. The percentage of seedless individuals in progenies ranges between 85 % (Spiegel-Roy *et al.* 1990) and 16.7 % (Tsolova *et al.* 1998), but when the rescue is performed in seedless x seedless crosses where the female and the male parent are obtained from a seeded x seedless cross or a seedless x seedless cross respectively, a high percentage of seedless progeny with natural big berries and with rather small seed traces is obtained (Perl *et al.* 2000).

The number of embryos obtained depends on the culture medium, the genotype of the parents and the date of harvest (Cain *et al.* 1983; Spiegel-Roy *et al.* 1985; Bouquet and Davis 1989; Emershad *et al.* 1989).

Gray (1992) observed severe ovular browning presumably due to tannins produced from wounding which diffused into the medium and formed a ring around each seed trace. To avoid this, every two weeks he excised the seed traces from the brown tissue and transferred it to a fresh medium. Tsolova and Atanassov (1994) also transferred undeveloped seed traces to a fresh medium after 12-14 weeks of culture. If tannins, phenolic or other toxic substances accumulate in the medium, abortion of the embryos would occur *in vitro*. Additionally, callus production would increase the excretion of metabolites. The development of calli from the outer integument was observed by Emershad and Ramming (1984), Gray *et al.* (1990) and Gribaudo *et al.* (1993). These calli are influenced by genotype, medium and genotype x medium (Gribaudo *et al.* 1993). Cv. Superior Seedless shows this behavior.

The principal objective of this work was to study the effect of medium culture when seed traces are cultivated for a long time. The period in which embryos can be excised is limited, and thus labor intensive. If the excision time would be prolonged these activities could be better organized. Embryo excision is generally performed after 60 - 90 d of culture (Emershad and Ramming 1984; Goldy and Amborn 1987; Gray *et al.* 1987; Bouquet and Davis 1989, Botta *et al.* 1992; Carreño *et al.* 1997) even though periods of 110 d (Valdez and Ulanovsky 1997), 160 d (Spiegel-Roy *et al.* 1985; Tsolova and Atanassov 1994) and 200 d (Cain *et al.* 1983; Agüero *et al.* 1996) have been tested. After cultivating seed traces *in vitro* for 8 months abnormal embryos were obtained by Agüero *et al.* (1996). This indicates that at late

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dates the quality of the embryos is lower. Some authors refering to the productivity of a cross use the terms transplantable plants (POMMER *et al.* 1995), obtained or established plants (BOUQUET and DAVIS 1989; GRAY *et al.* 1990), viable embryos (CAIN *et al.* 1983) or equivalent. The ISTA (International Seed Testing Association) percentage of germination is the proportion of seeds which have produced seedlings classified as normal (ISTA 1999). In this paper the terms normal plantlets, abnormal plantlets, contaminated embryos and embryos not developed are used to evaluate the results of rescued embryos. The percentage of germination is used according to ISTA rules.

Seed vigor is the total sum of properties of the seed which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence (PERRY 1987). Low vigor seeds usually have a slower mean rate of germination. In germination analyses of onion seeds it has been observed that lots with a high germination percentage at day 6 (first count), had a seedling field performance of higher agronomic quality (ORDOVINI *et al.* 1998). If this is true for stenospermic seeds, embryos able to germinate directly should present a higher proportion of normal plantlets, and a higher percentage of abnormal plantlets would be obtained from excised embryos. A second objective is to test this hypothesis.

Polyembryony in grapes was first reported by NEGRUL (1934) who observed twin seedlings in the Russian cultivar Nimrang. Studies with crosses between the seedless varieties Delight and Perlette were presented by MARISCALCO and CRESPAN (1995) underlining the potential contributions that polyembryony and somatic embryogenesis can offer for genetic improvement. Another objective is to evaluate the response of simple and multiple zygotic embryos to *in vitro* culture by observing the development of the plantlets.

Material and Methods

C r o s s e s : Crosses among seedless varieties were carried out in the collection of the Rama Caída Experimental Station, INTA, (San Rafael, Mendoza Province, Argentina) in November 1998. The crosses, the time from pollination to harvest and the percentage of germination are indicated in the first three columns of Tab. 1.

T a b l e 1

Crosses analyzed in the present work (in brackets the abbreviation). S. = Seedless; dph. = number of days from pollination to harvest. Seed trace put in culture (ST), total number of excised and directly germinated embryos obtained (TE) and total of normal embryos (NE) obtained at each date per cross. G %= Total percentage of germination (NE/ST x 100). NS, *: Differences significant and not significant according to the arcsine rule (5 %) for the number of embryos obtained at different dates of analysis

Cross	dph	G %		Day 90	120	150	180	210	240	
Blush S. x 73049	91	16	ST	90	75	89	75	90	90	
(Bx73)			TE	34	27	33	22	31	24	NS
			NE	13	10	15	12	14	17	NS
Bronx S. x Crimson S.	58	19	ST	90	90	75	90	90	84	
(BrxCr)			TE	23	34	23	27	21	14	*
			NE	13	27	15	21	16	5	*
Crimson S. x Malvinas S.	65	10	ST	90	75	90	45	90	90	
(CrxMl)			TE	23	25	25	8	27	35	NS
			NE	10	4	9	2	11	10	NS
Crimson S. x Serna S.	62	6	ST	90	90	92	84	92	92	
(CrxSe)			TE	30	29	27	17	17	16	*
			NE	13	4	8	2	4	4	*
Fantasy S. x Beauty S.	61	12	ST	90	75	75	75	75	90	
FxBe			TE	28	16	25	22	28	30	NS
			NE	10	6	8	8	11	14	NS
Ruby S. x Centennial S.	70	35	ST	90	90	90	90	90	90	
(RxC)			TE	42	62	43	51	46	53	NS
			NE	26	37	33	42	22	28	NS
Ruby S. x Dawn S.	67	34	ST	90	90	87	90	75	90	
(RxD)			TE	53	49	41	40	50	58	NS
			NE	26	32	28	26	34	33	NS
Superior S. x Dawn S.	43	14	ST	90	92	88	62	68	70	
(SxD)			TE	37	42	29	25	14	14	*
			NE	17	20	7	14	3	6	*
Total		18	ST	720	677	686	611	670	696	
			TE	270	284	246	212	234	244	
			NE	128	140	123	127	115	117	

In vitro culture: Berries were surface sterilized in 70 % ethanol (5 min) and sodium hypochlorite 1 % (10 min) and subsequently rinsed three times in sterilized deionized water. Seed traces were removed and cultivated in Petri dishes (9 cm diameter), 15 per dish in a Nitsch and Nitsch (1969) medium to which 2.7 $g \cdot l^{-1}$ of activated charcoal, 32 $g \cdot l^{-1}$ of sucrose and 2.4 $g \cdot l^{-1}$ of Phytagel (Sigma, St. Louis, USA) were added. The medium was also supplemented with IAA (3 $mg \cdot l^{-1}$) and GA_3 (5 $mg \cdot l^{-1}$). Once the axenic culture was established, seed traces were not transferred to fresh medium.

Thirty six dishes with each cross were chosen at random and were sorted into 6 groups. In some cases sample size was lower due to losses by contamination.

Directly germinated and excised embryos corresponding to each group were transferred at 90, 120, 150, 180, 210 and 240 (± 5) d of culture to Murashige and Skoog (1962) half diluted media. The medium was supplemented with 2.7 $g \cdot l^{-1}$ of activated charcoal, 25 $g \cdot l^{-1}$ of sucrose and 2.4 $g \cdot l^{-1}$ of Phytagel. All the cultures remained in a growth chamber at 24 ± 2 °C. Plantlets and excised embryos were placed under cold fluorescent light (16 h photoperiod).

Statistical analysis

Number of embryos per date: The number of embryos that resulted in normal plantlets at each date for each cross was analyzed by the χ^2 test. Differences at the 5 % level were compared with the Arcsin test (Tab. 1).

Evaluation of plantlets: Plantlets that germinated directly and excised embryos corresponding to each cross were evaluated after 30-45 d of culture and classified as normal, abnormal and not developed. The presence of multiple embryos was also computed. Eight cases of normal plantlets derived from previously classified abnormal multiple embryos were observed. These cases were considered normal for the statistical analysis.

Data corresponding to the evaluation of the embryos were analyzed by Correspondence Analysis (CA) with the package NTSyS (Rohlf 2002). CA was used because the data can be presented in a frequency chart. The variables taken into account were normality, abnormality, contamination and not developed. It is possible to estimate the data matrix from the combination of Tabs 1 and 2.

Polyembryony and direct germination: In excised or directly germinated embryos normality, abnormality, not developed and contamination were compared for each cross. These variables were also compared for simple and multiple embryos and CA was performed.

To asses if abnormalities were a product of a prolonged germination period or an intrinsic trait of the directly germinated embryos, the performance of directly germinated embryos was compared with respect to the extraction date (90, 120, 150, 180, 210 and 240 d) by means of χ^2 test (Tab. 2).

Results

Embryo rescue at different dates: No differences were observed in the crosses Bx73, FxBe, CrxMl, RxC, RxD (Tab. 1) indicating that the number of embryos for these crosses did not diminish over time. On the contrary, it occurred with the crosses BrxCr, CrxSe and SxD which showed a smaller number of embryos after 210 d of culture.

To observe the ultimate deadline possible for embryo rescue, data taken after 240 d of culture from embryos of the crosses CrxMl, RxC and RxD were analysed. Tab. 3 indicates that the total number of rescued embryos, their capacity to germinate and the number of transplantable plants obtained from CrxMl (340 d) and RxC (302 d) was not different from data obtained at 240 d. RxD presented a smaller number of embryos and transplantable plants without alteration of their capacity for germination.

Embryo development: Eighty four percent of the variability of data are explained by the two first correspondence axes. Points corresponding to each cross are clustered around two of the 4 variables: normality and abnormality. Two groups were determined: RxD, RxC, Bx73 and BrxCr that relate to normality and CrxSe, CrxMl, SxD and FxBe that relate to abnormality. The relative frequency of the variable normality (0.466) was somewhat higher than abnormality (0.444), but both characterize the structure of the data. The relative frequency of the individuals (a cross for each date tested) was uniform: 0.0208.

Table 2

Number of directly germinated embryos after 90-240 d and evaluation of their development 30 d after transfer to MS media. N: Normal plantlets; A = Abnormal plantlets. *P* (χ^2) of the comparison of normal plantlets among dates for each cross

	Day												
	90		120		150		180		210		240		*P*(χ^2)
	N	A	N	A	N	A	N	A	N	A	N	A	
Blx73	1	4	0	0	2	4	1	3	3	3	4	1	0.284
BrxCr	0	0	2	1	0	0	2	1	1	1	1	5	0.375
CrxMl	0	1	1	1	3	4	0	0	7	9	9	20	0.810
CrxSe	0	0	0	0	0	0	0	6	2	6	0	8	0.128
FxBe	1	0	2	4	3	7	7	9	8	10	12	13	0.765
RxC	0	0	0	0	6	1	4	7	5	16	13	11	0.216
RxD	1	1	3	3	4	4	6	4	10	8	12	7	0.984
SxD	1	3	3	6	2	2	6	6	1	7	3	2	0.469

Table 3

Embryos excised after 240 d of in vitro culture. ST: seed traces sown; EE: excised embryos; NE: normal embryos; NG: excised embryos that did not germinate; DG: directly germinated embryos. *, NS: Significant and Not significant at the 5 % level according to arcsin rule

	d	ST	EE/ST		NE/ST		NG/EE		DG/EE	
CrxMl	240	90	0.39	NS	0.11	NS	0.00	NS	0.83	*
CrxMl	340	60	0.32		0.08		0.05		0.58	
RxC	240	90	0.59	NS	0.31	NS	0.06	NS	0.45	NS
RxC	302	120	0.54		0.26		0.05		0.48	
RxD	240	90	0.64	*	0.37	*	0.05	NS	0.33	NS
RxD	280	135	0.50		0.19		0.03		0.37	

Polyembryony: The primary data matrix used for CA is presented in Tab. 4 and the projection of variables in the first two axes is presented in Fig. 1. These axes represented 98.65 % of the sample variability. As in the analysis of embryo development, the most important relative frequency was that of normality (0.503) and abnormality (0.411), both characterizing the structure of data. It was observed that polyembryos of SxD are drawn towards normality (58 %) but when single embryo development was analyzed, the cross was drawn to abnormality. On the contrary, polyembryos of Bx73 are drawn towards abnormality (65 %). In Fig. 1 a tendency of simple embryos of crosses CrxMl and CrxSe toward this variable is observed. Single embryos of these crosses behaved inversely. Two groups for both single or multiple embryos were formed with the remnant crosses: RxC, RxD and BrxCr that clustered around normality and CrxMl, CrxSe, FxBe which clustered around abnormality. Germination capacity of multiple embryos was higher in all cases with the exception of BrxCr where no differences between simple or multiple embryos were observed.

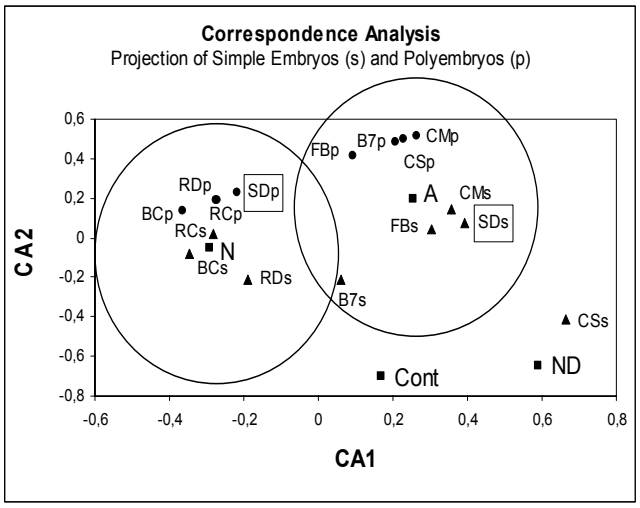

Fig. 1: Crosses projection and analyzed variables (N = normality; A= abnormality; Cont. = contaminated embryos and ND = not developed embryos) on the first two axes of the analysis of CA when simple and multiple embryos are analyzed. E.g., RDs = RxD simple embryos, RDp = RxD polyembryos. Tendency towards normality of polyembryos of SxD is framed. Groups are encircled.

Table 4

Primary data matrix for CA of the result of simple (S) or multiple embryos (M). PE % = Percentage of polyembryony. A: abnormality; N: normality; ND: not developed embryos; Cont.: contaminated embryos. Excised embryos or directly germinating plantlets that became abnormal, normal, stopped their development or became contaminated when they were sown in MS medium

Cross		A	N	ND	Cont.	PE %
Bx73	M	17	9	0	0	15
	S	52	72	17	4	
BrxCr	M	5	10	0	0	11
	S	33	87	6	1	
CrxMl	M	24	11	0	0	24
	S	62	35	11	0	
CrxSe	M	24	12	0	0	26
	S	49	23	24	4	
FxBe	M	43	30	0	0	49
	S	40	27	8	1	
RxC	M	13	21	0	0	11
	S	85	167	7	4	
RxD	M	10	16	0	0	9
	S	73	163	22	7	
SxD	M	27	38	0	0	40
	S	55	29	10	2	
Total	M	163	147	0	0	21
	S	449	603	105	23	

Crosses with the highest capacity to produce multiple embryos were SxD (40 %) and FxBe (48 %), which was only advantageous for SxD because, as mentioned above, polyembryos produced more normal plantlets. The above mentioned 8 cases of abnormal plantlets that reverted to normal plantlets belong to the SxD cross. FxBe did not present differences among single and polyembryos: both single and polyembryos of FxBe approach to abnormality.

Direct germination: The number of normal and abnormal plantlets of directly germinated embryos obtained is presented in Tab. 2. Differences were not observed at the date of Petri dish opening, indicating that the abnormality of plantlets was independent of the timing of this event and of the length of time used for embryo culturing.

The primary data matrix used for the correspondence analysis resulting from excised and directly germinated embryos is presented in Tab. 5. The small contribution of directly germinated embryos compared with those excised is evident: 80 % ((598/(152+598))*100) of normal plantlets came from excised embryos and 57 % of directly germinated embryos produced abnormal plantlets as compared to 36 % of abnormal plantlets produced by excised embryos. The first two axes of correspondence explain 93 % of the variability. Data are mainly characterized by the variables normality and abnormality as was discussed above. In Fig. 2 analyzed variables are plotted together with the crosses that are separated between those coming from excised embryos and those germinated directly. Plantlets coming from the directly germinated embryos clustered around abnormality, except for RxD which is drawn towards normality. The excised embryos form two groups: those that tend to abnormality (CrxMl, CrxSe, FxBe and SxD) and those that tend to normality (Blx73, BrxCr, RxC and RxD). These groups coincided with those already defined in the global study of data but it is interesting to note that directly germinated embryos of crosses that tend to normality also tend to abnormality.

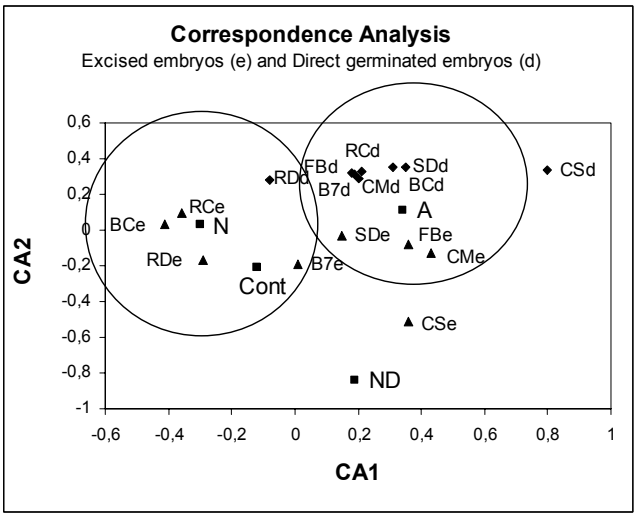

Fig. 2: Crosses projection and analyzed variables on the first two axes of the CA when excised or direct germinated embryos are analyzed. Groups are encircled. E.g., RDd = RxD Direct Germinated embryos; RDe RxD Excised Embryos. For abbreviations see Fig. 1.

Table 5

Primary data matrix for CA of the result of excised and directly germinated embryos. The rate of direct germination for cross (DGR) is presented. Excised embryos (EE) or directly germinating plantlets (DG) that became abnormal, normal, stopped their development or became contaminated when they were sown in MS medium. For abbreviations see Tab. 4

Cross		N	A	ND	Cont.	DGR
Bx73	DG	11	15	0	1	0.14
	EE	70	54	17	3	
BrxCr	DG	6	8	0	0	0.06
	EE	91	30	6	1	
CrxMl	DG	20	35	0	0	0.43
	EE	26	51	11	0	
CrxSe	DG	2	20	0	2	0.06
	EE	33	53	24	2	
FxBe	DG	33	43	0	1	0.58
	EE	24	40	8	0	
RxC	DG	28	35	0	0	0.15
	EE	160	63	7	4	
RxD	DG	36	27	0	0	0.20
	EE	143	56	22	7	
SxD	DG	16	26	0	0	0.24
	EE	51	56	10	2	
Total	DG	152	209	0	4	0.20
	EE	598	403	105	19	

There was a linear increase of directly germinated embryos between the first and the last date, except for the cross Bx73, where the germination frequency was 0.15, 0.00, 0.21, 0.18, 0.19 and 0.21 (Tab. 2) corresponding to the first frequency for 90 d and the last one for 240 d. The highest frequency of germinated embryos was observed after 240 d which corresponded to the crosses CrxMl and FxBe (0.83). The plantlets from direct germination had more capacity to generate multiple embryos than the excised embryos with the exception of SxD. The correspondence analysis allowed us to represent and to summarize the crosses among seedless grapevines as they cluster near physical objects (crosses) on the axes of correspondence. This method also allowed us to summarize the data referring to the properties of the conformed groups rather than each particular cross.

Discussion

After 240 d of culture there were no differences in the number of embryos obtained in 5 of 8 crosses. There were no differences in two crosses analyzed after more than 300 d in culture in the same Petri dish. This observation, for those particular crosses, rejects the supposed generalized toxic effect by the diffusion of phenolic or tannic substances produced after catabolism of the seed traces.

Superior Seedless and to a lesser extent Bronx Seedless do not present hard seed coats like Blush Seedless or Ruby Seedless. They have, on the contrary, a callus from the external layer of the seminal coat which spreads over two or three times the original surface of the seed trace. This callus probably accumulates toxins in the culture media that overcome the absorption capacity of the added activated charcoal. For this reason, it is probable that Superior Seedless and Bronx Seedless had fewer surviving embryos at 240 d. Activated charcoal absorbs toxic substances and gradually liberates hormones in culture media (Evert and Taylor 1990, Hornung 1997). It has also antioxidative effects and is incorporated into the medium to prevent or reduce oxidative browning caused by phenolic oxidation. It has been observed in grapevines that the addition of activated charcoal increases the survival rate of embryos obtained (Bouquet

and DAVIS 1989, GRIBAUDO *et al.* 1993), and decreases tissue browning, callusing and medium discoloration (CAIN *et al.* 1983).

Two groups of crosses which coincided in the embryo development resulted in normal plantlets, mosty produced by RxD, RxC, Bx73, and BrxCr while CrxSe, CrxMl, SxD, FxBe produced mostly abnormal plantlets. An exception was observed in the development of multiple embryos of SxD (normality) and of Bx73 (abnormality). A coincidence was observed in the behaviour of the female cultivars RxD and RxC and the cultivars CrxSe and CrxMl without pollinator influence upon the normality/abnormality trait. As was stated by MARISCALCO and CRESPAN (1995) polyembryony might depend on genotype RxD <RxC <BrxCr <Bx73 <CrxMl <CrxSe <SxD <FxBe. Polyembryony is advantageous to diminish the number of abnormal plantlets in the last two crosses by successive transfer of embryos to fresh media. This suggestion is based on the observation that a larger number of normal plantlets of polyembryonic origin can be obtained in SxD and that polyembryos presented a greater germination capacity in this cross.

Direct germination is not recommended since most of the normal plantlets (80 %) came from excised embryos. The final percentage of plantlets from directly germinated embryos after 240 d was 24.5 %, but an abnormality of 57 % was found. Moreover, the fact that a plantlet develops abnormal structures is independent of the time it remains in a Petri dish. The analyses performed in this study on directly germinated plantlets discredits the hypothesis that plantlets able to germinate directly are more vigorous. However, the observations allow us to optimize the handling of seed traces of Blush Seedless. No embryos germinated after 90 d of *in vitro* culture. These plantlets start their development in Petri dishes by increasingly consuming nutrients and eliminating secondary metabolites. The most harmful action of directly germinated plantlets is the physical pressure they exert on the Petri dish, causing it to open and allowing the entry of environmental contaminants. It is suggested to remove the 90-d-old germinated plantlets from the Petri dish and to excise the embryos from the remaining seed traces. Petri dishes without germinated embryos can wait for embryo excision up to 240 d.

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