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Influence of size of culture vessel on in vitro proliferation of grape in a liquid medium

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Abstract. The in vitro proliferation of *Vitis vinifera* L. cv. Liemberger in a liquid medium was compared in 125 and 250 ml Erlenmeyer flasks and in 473 ml (pint) Mason jars. After 6 weeks of culture the jars yielded a significantly greater number of shoots 3 mm or longer than the flasks. Jars yielded the greatest number of shoots 7 mm or longer, followed by 250 ml, then 125 ml flasks. The mean length of shoots in the 250 ml flasks was significantly greater than that of shoots in 125 ml flasks. The final mean fresh weight of the cultures in jars was significantly less than that of the cultures in flasks. Thus the size of vessel used influenced the in vitro proliferation of grapevines in liquid culture.

Introduction

Much attention has been focused on establishing optimum inorganic salt, vitamin, growth regulator and other chemical requirements for maximum shoot proliferation of tissue cultured plant material [3]. Some culture conditions such as temperature and illumination have been studied [5] and it was recently shown that the concentration of agar in a culture medium influences the rate of in vitro proliferation of *Malus* sp. 'Almey' and *Pyrus communis* 'Seckel' [8]. The use of liquid media results in increased growth and proliferation, as compared to agar-gel media, for *Nicotiana tabacum* cv. Xanthi-nc [11], *Fuchsia hybrida* cv. Swingtime [10] and *Vitis vinifera* cultivars [4].

Another physical parameter which appears to influence proliferation is the size of the culture vessel. Adams [1] reported that strawberry shoots rooted within 2 months in a liquid medium in small containers, whereas the process was greatly accelerated on the same medium in larger vessels. We observed that the proliferation of *Vitis vinifera* cv. Liemberger in a liquid medium was influenced by the size of the culture vessel. The present investigation was conducted to determine the effect of culture vessel size on shoot number, length and fresh weight of excised shoot tips of 'Liemberger'.

Materials and methods

Three cm shoot tips from *Vitis vinifera* cv. Liemberger were cut from plants grown in a shadehouse. Expanded leaves were removed and shoot tips were

surface sterilized by vigorous stirring for 20 min in a 0.6% solution of commercial sodium hypochlorite containing 0.1% Tween 20. Shoot tips were rinsed 3 times in sterile distilled water, aseptically trimmed to 2–4 mm and placed on an initiation medium. For the initial establishment of shoot tips in culture, Murashige and Skoog (MS) medium [6] was diluted to 3/4-strength and supplemented with (per liter) 60 mg adenine sulfate, 128 mg $NaH_2PO_4 \cdot H_2O$, 0.023 mg indole-3-butyric acid (IBA), and 2 mg N^6-benzylaminopurine (BAP). This medium was adjusted to pH 5.7, 0.7% Difco Bactoagar was added and 15 ml was dispensed into 25 mm x 150 mm culture tubes capped with polypropylene closures (Bellco Kaputs). Six weeks after initiation the cultures were transferred to proliferation medium. For the proliferation of grapevine cultures MS medium was used at full strength and supplemented with (per liter) 80 mg adenine sulfate, 170 mg $NaH_2PO_4 \cdot H_2O$, 0.03 mg IBA, and 2 mg BAP. This medium was adjusted to pH 5.0, 15 ml was dispensed into 125 ml Erlenmeyer flasks and these were capped with aluminium foil. Both agar-gel and liquid media were autoclaved at 121°C for 15 min at 1.4 atm (142 kPa).

Cultures were maintained at 23 ± 2°C with a 16-h photoperiod under 48 (agar medium) or 30 (liquid medium) $\mu Em^{-2}s^{-1}$ (400–700 nm) provided by cool-white fluorescent lights (Vita-Lite Power Twist; Duro Test Electric Ltd., Ontario). The liquid cultures were placed on a device that tilted the flasks in opposite directions, 30° from the vertical every 30 s.

Proliferating cultures were transferred into fresh liquid medium every 2 weeks. Twelve each of 125 ml and 250 ml Erlenmeyer flasks and 473 ml (pint) Mason jars each received 7.5 ml of grapevine proliferation medium. They were covered with aluminium foil and autoclaved. One 2 cm long shoot excised from actively proliferating cultures was weighed aseptically and placed in each vessel. These cultures were transferred in toto into fresh medium every 2 weeks. No trimming was carried out at the time of transfer. There was no need to excise dead tissue, as very little was observed and leaves with aberrant morphology were left on so that the weight data were not distorted. After 4 or 6 weeks of proliferation in the test vessels, 6 randomly selected cultures were removed from each vessel type, blotted dry with a paper towel and weighed. Shoots 3 mm or longer were counted and their length was measured. Means were analyzed by analysis of variance (Anova, $p = 0.05$), followed by Student-Newman-Keuls (SNK) Multiple Range Test ($p = 0.05$) [9].

Results and discussion

After 4 weeks in culture, the mean number of shoots 3 mm or longer harvested per vessel did not differ significantly among the 3 types of culture vessels (Table 1). After 6 weeks, however, the mean number of such shoots harvested from jars was approximately twice that harvested from either size

Table 1. Mean number and length of shoots harvested after 4 or 6 weeks in various sizes of vessels

Vessel type and size	Mean number of shoots harvested per vessel*			Mean length*	
	4 weeks (≥ 3 mm)	6 weeks		4 weeks	6 weeks
		(≥ 3 mm)	(≥ 7 mm)		
125 ml Erlenmeyer	4.3a	5.2b	2.8c	7.4a	7.5b
250 ml Erlenmeyer	3.3a	5.3b	5.2b	6.9a	9.9a
473 ml Mason jar	4.2a	10.5a	7.7a	6.7a	9.3ab

*Values are the means of 6 replicates for the 4-week harvest with Mason jars, where one jar was lost due to contamination. Means within columns not followed by the same letter are significantly different. (Anova, $p = 0.05$; SNK, $p = 0.05$).

Erlenmeyer flask. Also, the number of shoots 7 mm or longer was significantly different in all 3 types of vessels. The jars produced more shoots than the 250 ml flasks which, in turn, yielded more shoots 7 mm or longer than the 125 ml flasks. Shoots 7 mm or longer are more easily manipulated and are routinely excised in this laboratory for transfer to rooting media. In addition the mean number of shoots 3 mm or longer increased significantly between 4 and 6 weeks in jars but not in either flask. Thus the size of culture vessel influenced the differentiation of the tissue cultured material into shoots.

The mean length of shoots harvested from all culture vessels was not significantly different after 4 weeks in culture (Table 1). After 6 weeks, shoot length in the 250 ml flasks was greater than in the 125 ml flasks. The mean shoot length at 6 weeks was not significantly different than at 4 weeks in any of the vessel types tested.

The mean fresh weight of the cultures after 4 weeks was not significantly different in the 3 sizes of vessels tested (Table 2). After 6 weeks, however, the mean fresh weight of the cultures in the jars was less than that of the cultures in the flasks. Although the cultures in jars increased less in weight than those in flasks they produced a greater number of shoots. The weight increase of the cultures in flasks was largely due to callus formation and growth rather than to shoot proliferation. Thus the use of jars resulted in a very favorable differential in the production of usable shoots, in comparison to the use of either size flask.

The observed differences in proliferation can be attributed to the size of vessel used because: a) the explants were all obtained from the same source; b) their initial weights were not significantly different ($p = 0.05$) and c) they were maintained in an identical culture environment. In the studies on the effect of agar concentration on shoot proliferation [8] the author acknowledged the possibility that the observed effect was at least partly due to the presence of inhibitory contaminants in the commercial agar preparations.

Table 2. Initial and final mean fresh weight of the explants from various sizes of vessels

Vessel type and size	Fresh weight (g)[a]			
	4 weeks		6 weeks	
	w_i[b]	w_f	w_i	w_f
125 ml Erlenmeyer	0.06a	3.50a	0.06a	7.44a
250 ml Erlenmeyer	0.09a	3.01a	0.05a	7.93a
473 ml Mason jar	0.05a	3.10a	0.07a	5.91b

[a]Values are the mean of 6 replicates except for the 4-week harvest with Mason jars, where one jar was lost due to contamination. Means within columns not followed by the same letter are significantly different (Anova, $p = 0.05$, SNK, $p = 0.05$).
[b]w_i = initial weight; w_f = final weight

Similarly, the possibility that the different sizes of culture vessels used here might contribute different amounts of inhibitors to the culture media cannot be eliminated, because the flasks were of Pyrex and the jars were not. One observation which weighs against this interpretation, however, is the difference in the mean number of shoots 7 mm or longer harvested after 6 weeks from the 125 ml and 250 ml Erlenmeyer flasks (Table 1). Alternatively, the jars may have released a stimulator. While the present experiments do not demonstrate that the size of the vessels is necessarily the only factor responsible for the differences in proliferation they do indicate that size is important. The increased proliferation observed in jars may be due to better aeration of the medium, a greater availability of nutrients to the cultures and a more efficient removal of toxic metabolic wastes from the immediate surroundings of the cultures. These would be consequences of the different fluid circulation patterns in the different culture vessels due to their different dimensions.

It is also possible that the differences in proliferation observed with the different size vessels were due to crowding. It is conceivable that the surface area available to the culture may to some extent regulate the number of axillary shoots which can develop. While it is well recognized that in vitro shoot proliferation is largely determined by chemical factors, i.e., the cytokinin: auxin ratio, there is no a priori reason to believe that a physical factor such as the available surface area could not superimpose a modulation onto the growth response of the cultures. This view is supported by the observation that the differences in the number of shoots 3 mm or more in length and in the fresh weight of the cultures in the different size vessels were not apparent at 4 weeks but were quite evident at 6 weeks. Cultures in flasks may have experienced stress due to crowding between the fourth and sixth week. Cultures in jars would not yet have been subject to the same constraint.

Since the volume of air above the liquid medium is different between the various vessel sizes, the differences in proliferation may reflect differences in gas exchange between the liquid medium and the atmosphere within the vessels. The gas atmosphere above plant tissue cultures is known to contain various volatiles including ethylene [12]. This regulatory compound can either stimulate or inhibit fresh weight increase in vitro [5, 7]. The differences in proliferation observed in the present experiment may result from an ethylene effect.

No difference was observed between the different vessel sizes with respect to plant quality. The aberrant growth mentioned above consisted of large dark green leaves with distorted shapes and a very irregular surface. Some fasciation of shoots was also present. Most of the cultures contained some of these aberrations after 6 weeks but there was no noticeable difference in the degree of aberration from one size of vessel to the next. This is in agreement with a previous report indicating that vitrification was not affected by the volume of the containers utilized [2]. In this laboratory proliferating grapevine cultures maintained in liquid medium without reculturing became vitrified within four weeks. The leaves became shiny and translucent and the stems of the axillary shoots were very brittle. However, shoots excised upon reculturing at 14 day intervals and placed onto rooting medium developed roots within 7 to 14 days with nearly 100% success [4].

It is clear from the data presented in this report that the size of culture vessel influences the proliferation of shoots in liquid media. Operators of commercial tissue culture laboratories may wish to take note of this in order to maximize their production. The determination of optimal vessel size for the culture of any plant species would of course have to take into consideration the size of the initial subculture and the reculture interval as these factors bear directly on nutrient supply, toxic waste buildup and crowding.

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