VITRIFICATION OF PLANTS CULTURED *IN VITRO*

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The term "vitrification" describes morphological and physiological disorders of plants cultured *in vitro*. The descriptions of vitrified cultures given by various authors are very similar whatever the species involved. Stems are thickened and translucent, leaves thick, brittle, wrinkled, curled, and frequently very elongated, with no differentiated palisade tissue (Figure 1). There is a general hyperhydricity of the cells as well as a deficiency of chlorophyll, and usually the lignification of vessels and tracheids is defective (18). Such cultures lose all capacity for reproduction and may threaten the continuance of their clones. The problem is serious; most micropropagation laboratories face vitrification of their cultures and many tissue culturists have focused their efforts on practical means of avoiding vitrification (Table 1).

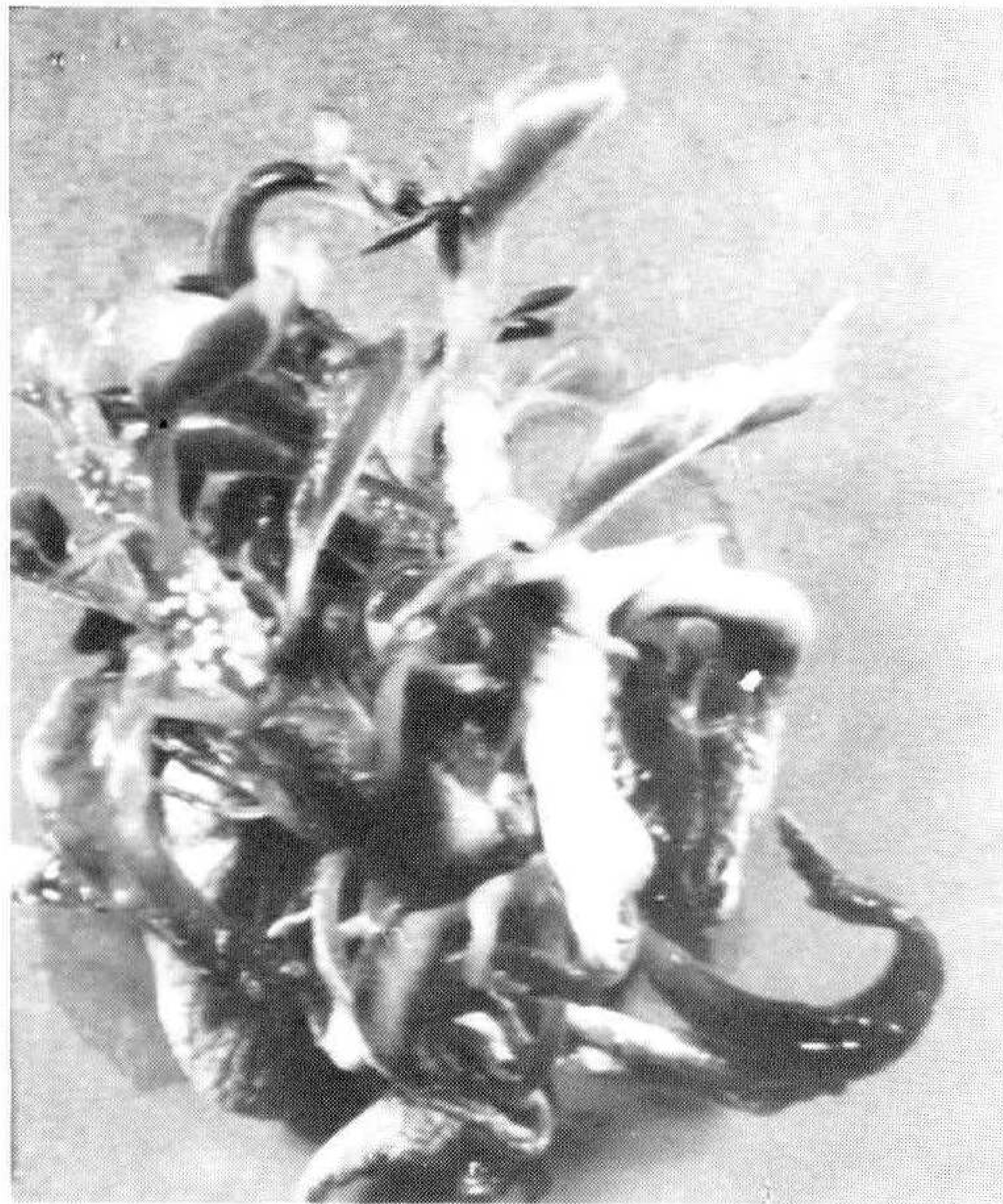

Figure 1. Vitreous growth of 'Spur MacIntosh' apple on 1 g/liter Gelrite.

Though numerous correlated factors are doubtless involved in this many-faceted phenomenon, the concentration and brand of agar, the level of benzyladenine (BA), and the content of ammonium ions are the main contributors of occurrence of vitrification.

Table 1. Percentage of vitrified shoots in four Italian micropropagation laboratories (Zanzi Vivai, Vitro Plant, Battistini, Vitro Coop). Mean over one year of cultures.

Percent of Vitrified Shoots			
Rootstocks		Species and Cultivars	
M 26	5 to 30	Peach	5 to 100
GF 677	5 to 30	Pear	30 to 40
GF 655/2	2 to 10	Apple	5 to 30
GF 43	2 to 30	Kiwifruit 'Tomuri'	20
CAB 6P	5 to 30	Kiwifruit 'Hayward'	30
CAB 11E	5 to 30	Chestnut	50
		Filbert	20

The concentration and brand of agar

The occurrence of vitrification is an agar-related problem. Agar should not be considered simply as a means of solidifying culture media. Both the concentration and brand of agar affect the chemical and physical characteristics of a culture medium (4). Moreover there are marked differences in nutrition composition among different agar brands (15), and striking variations in the solidity of gels among similar concentrations (4).

Tissue culture studies in recent years have demonstrated that agar concentrations have a strong influence on the growth and development of various explants (13, 16, 17, 19). Shoot growth and shoot proliferation of *Malus* 'Almey' and *Pyrus communis* 'Seckel' were significantly influenced by agar levels (14).

The incidence of vitrification could be lowered by raising the level of agar in the culture medium but, in so doing, shoot proliferation was reduced (5, 7). With globe artichoke, increasing the agar concentration from 0.6% to 1.1% eliminated shoot vitrification but halved shoot proliferation rate (Table 2). Debergh *et al.* (5) attributed this result to the matric potential, for which the agar would be responsible. Similar results were obtained with 'Gala' apple using Gelrite plus Sigma-agar as a gel (11).

Table 2. Influence of different concentrations of Difco Bacto-agar on percentage of vitrification and proliferation ratio. Partial data from Debergh *et al.* (5).

	Percent of Vitrified Shoots	Propagation Ratio
BM	60 to 90	3.75 a
BM + Agar, 0.6%	75 to 100	3.30 a
BM + Agar, 0.9%	20	1.90 c
BM + Agar, 1.1%	0	1.50 cd
BM + Agar, 1.5%	0	1.20 d
BM + Agar, 2.0%	0	1.00 d

Values followed by the same letter do not differ at $P \leq 0.05$ (Letter b is on data not reported).
BM = basic medium

As shown in Figure 2, the incidence of vitrification is lowered by increasing either Sigma-agar or Gelrite content. In this study no vitrification was produced by combinations of 1 to 1.5 g/liter Gelrite plus 2 to 4 g/liter agar.

Regarding agar brand, vitrification has sometimes been overcome by substituting one gelling agent for another. For example, apple cultivars become vitreous when Gelrite or Phytagar was used to solidify the proliferation medium (12), but not with Difco Bacto-agar. Gelrite is used commercially for *in vitro* propagation of some ornamental plants and of paradox walnut rootstock (6), but it induces vitrification with a lot of fruit plants (R. H. Zimmerman, personal communication). To prevent vitrification of their cultures, private laboratories test the quality of each stock of agar in a small number of jars before using it in large quantities.

The question is: how do the concentration and brand of agar influence the characteristics of the medium and thus vitrification?

First of all, with increased agar concentrations, the medium tends to be firmer, losing the characteristic fluid consistency typical of lower concentrations; the availability of water is diminished and the diffusion of macromolecules is restricted (13). Water is generally recognized as a key-point in vitrification; affected plants in fact have a greater diffusion of water into the cells. The reduced availability of water and of other components may lead to the inhibition of both vitrification and shoot proliferation.

Secondly, the chemical and physical properties of each agar are specific, so that one species may be influenced by such properties while others are not.

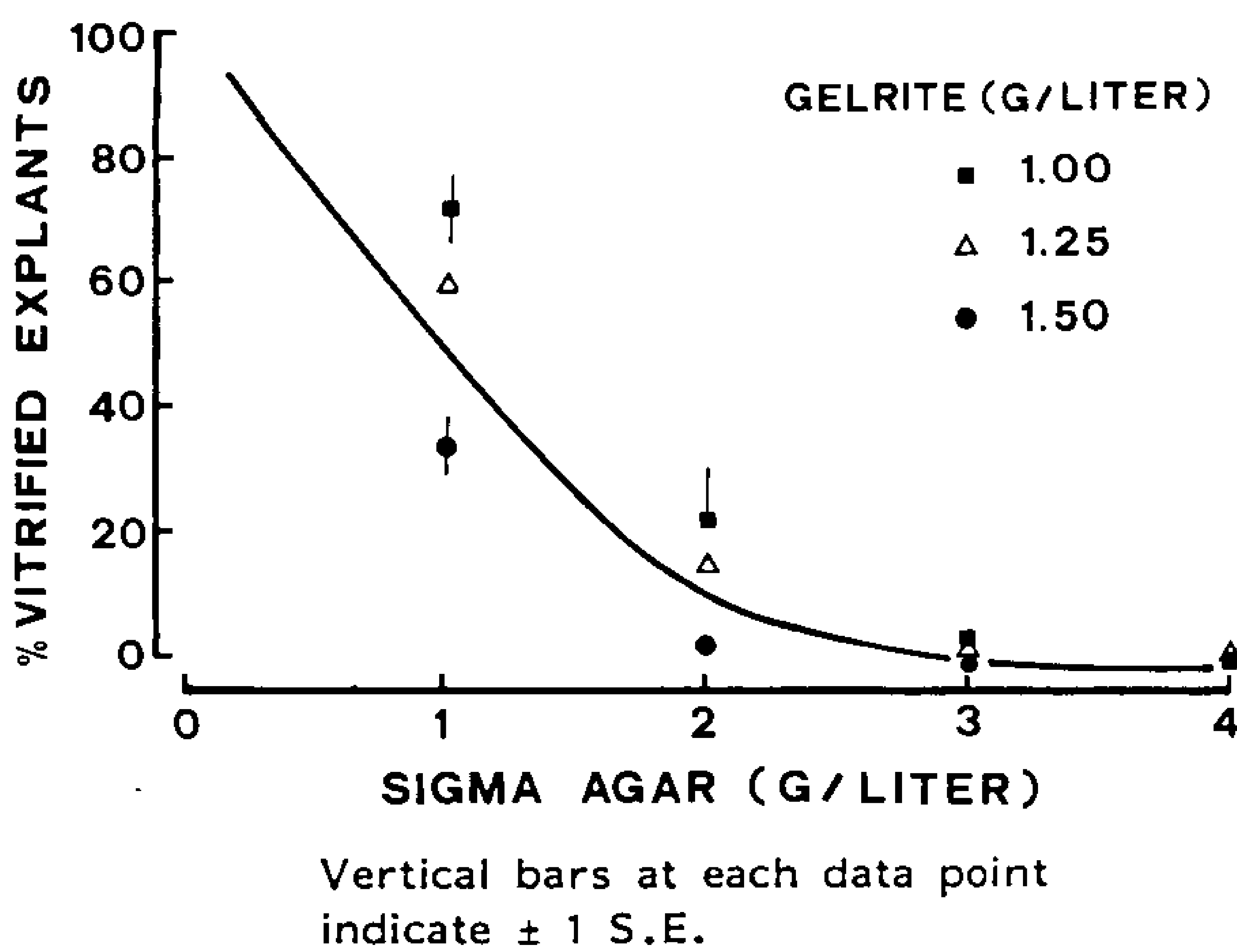

Figure 2. Effect of Sigma-agar concentration on percentage of vitrified explants at different concentrations of Gelrite. The curve indicates the trend of the phenomenon. Partial data from Pasqualetto *et al.* (11).

The level of BA

The basis of micropropagation is the stimulation of new shoots *in vitro* by treatment with an appropriate plant growth hormone. A cytokinin in the culture medium stimulates growth of axillary and/or adventitious buds.

Debergh (4) found that vitrification was influenced with BA levels at low agar concentrations. Bornman and Vogelmann (2) reported a high incidence of vitrescence in *Picea* sp. when gel media of low rigidity were used and, in particular, a highly significant negative correlation between the uptake and accumulation of 14C-labelled BA and the stiffness of the gel.

Vitrification of 'Gala' apple cultivar cultured on Murashige-Skoog medium (10) proved higher with 4.4 uM of BA than with 2.2 uM of BA (11). The vitreous condition in apple cultures can sometimes be overcome by transferring them from a medium containing BA to one with 2iP or no cytokinin at all (20). However, by reducing cytokinin levels in the medium, or changing type, we can limit or completely solve the problem of vitrification, but at the cost of lowering or halting shoot proliferation. On the other hand, it seems that the effect of BA on vitrification can be overcome by increasing gelling agent concentration, so that the effect occurs only at particular concentrations of the agar. Figure 3 shows clearly that at 0.5 to 1 g/liter agar the frequency of vitrification is higher than at 3 or 4 g/liter agar, but in both cases the concentration of BA makes little or no difference while at the other agar concentrations the BA level is influential.

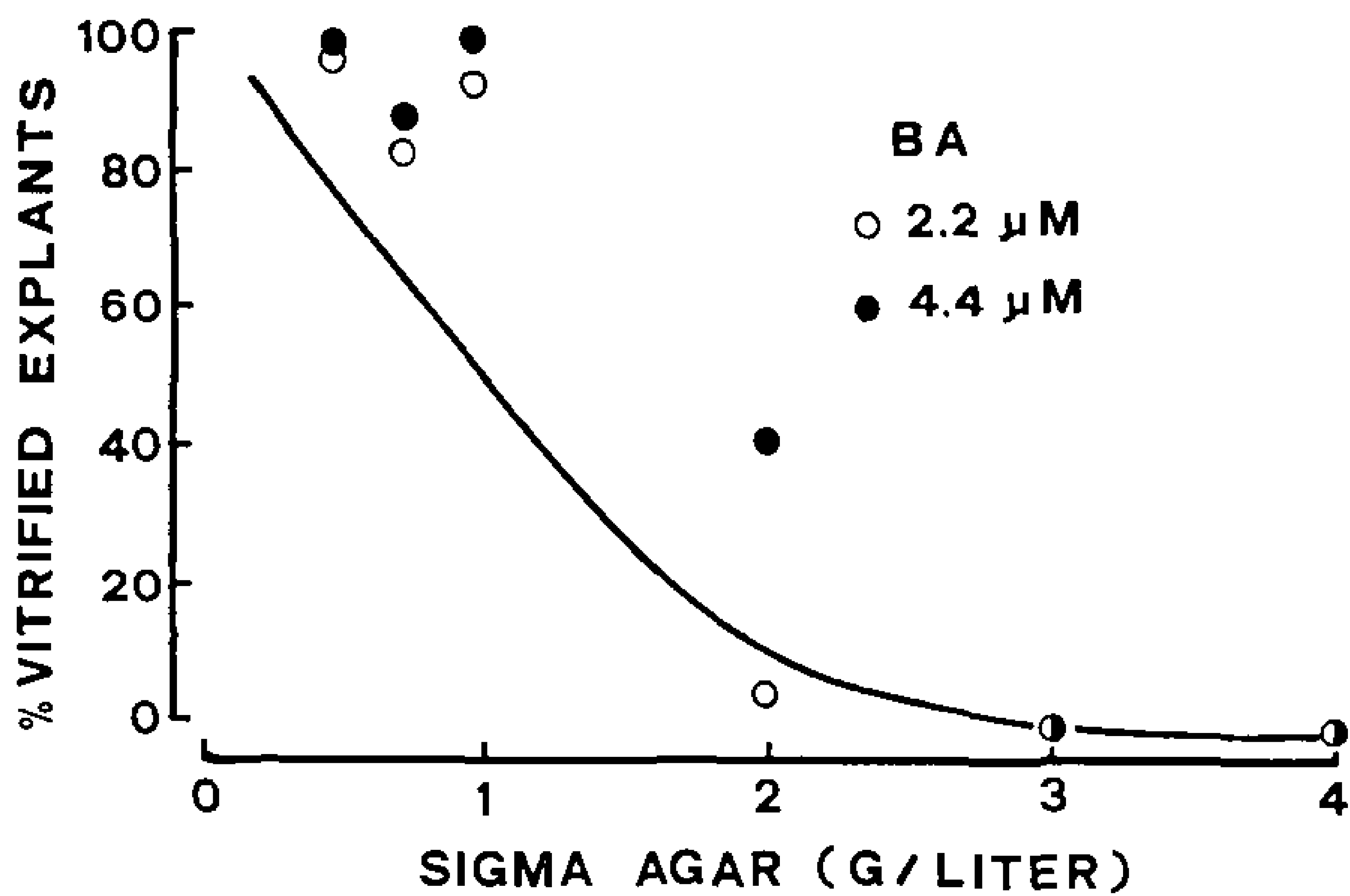

Figure 3. Effect of two levels of BA on percentage of vitrified explants. The curve indicates the trend of the phenomenon in relation to gelling agent concentration. Partial data from Pasqualetto *et al.* (11).

The content of ammonium ions

One salient feature of the syndrome is that most reported cases of vitrification have occurred using Murashige and Skoog's medium (10), which has a particularly high content of ammonium nitrate

(18). With *Salix babylonica*, vitrification of shoots was reduced by varying the quantity of nitrogen in the culture medium (3).

Vitrified shoots were produced in multiplication cultures of *Castanea sativa* when Murashige and Skoog medium was used in the subcultures, whereas normal shoots were obtained when Heller's macronutrient formula was used, with or without addition of 1 mM ammonium sulphate (18).

Two basic facts regarding the vitrification phenomenon are generally recognized: firstly, the reduction of lignin and cellulose content of tissues and, secondly, the enlargement of cells. One possible correlation between these two basic facts and ammonium ions has been suggested in the literature. Ammonium ions are assimilated faster than other nitrogen sources such as nitrates. Both ammonium ions and the lignin synthesis pathway need carbohydrates, so that a rapid uptake of the former may divert carbohydrates from the latter (1). Deficiency of lignin and cellulose, in fact, results from a decreased C/N ratio produced by an excess of N (9). Both deficiencies would tend to reduce wall pressure and so favour increased absorption of water with a consequent enlargement of the tissues.

CONCLUSIONS

Although growth room temperature and light sources cannot be ignored, agar, BA, and ammonium ions are more often described as factors able to eliminate the vitreous condition in cultures.

The availability of water in the culture jars seems to be a keypoint of the problem and agar, BA, and ammonium ions are in some way related to it. Increasing agar concentration reduces the availability of water and the translocation of macromolecules. Each brand of agar influences the chemical and physical characteristics of the medium in a specific way and consequently the water status in the jar. Cytokinins can cause an increase in the size of leaf tissues by a process involving only cell enlargement (8), while rapid ammonium ion uptake brings about a drop in the C/N ratio, leading to a deficiency of lignin and cellulose with a consequent absorption of water into the cells.

On the other hand, vitrification can occur in one laboratory and not in another even when both use the same techniques of *in vitro* culture and even the same plants (4).

A solution to this problem, and especially one able to maximize shoot proliferation and minimize vitrification, will require simultaneous attention to a number of different factors, including those considered above.

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