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The use of silica sand in micropropagation of woods

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ABSTRACT: Cultures *in vitro* made in agar are rather precarious, because gel strength varies both with the medium formula used and the source and grade of agar. Any solidifying agent (like for example agar) should be strong enough to support cultivated plantlets, yet liquid enough to allow the nutrients and drossy products from plants through the medium. It should also be a chemically inert material. Agar, especially in acid solutions, is an undefined constituent of culture media, namely in the mentioned properties. Silica sand, used in cultures of herbs up to the present time, is applicable also in cultures of *Sorbus sudetica*. The required acid medium is exactly defined if sand is substituted for agar. Similar cultures of wood species, including conifers, will be realized in future research.

Keywords: micropropagation; agar; silica sand; acid medium; *Sorbus sudetica*

Agar is used for the micropropagation of wood species *in vitro* as a support whereby nutrient media are solidified. Agar is used in 6–8 g/l concentration to be solid enough to support cultivated plantlets, yet liquid enough to allow the nutrients and drossy products from plants to pass through the medium. Agar, a natural polysaccharide containing D- and L-galactose partly esterified by sulphuric acid, has however a disability range. It is considered a chemically undefined substance, which is why media with agar are just partially defined. Properties like solidity and adulterants vary in origin and by the technique of its factory processing. Agar gel becomes liquid during sterilization in an autoclave as a result of hydrolysis, namely more in media with higher content of salts and phytohormones than in less concentrated media. It is also more unstable with pH 4.5 than by pH 5.7. The liquidity then has an impact on the generation of plantlets. Development of plantlets is affected by the hydrolyzed non-solid agar. It can induce vitrification, namely in media containing a high cytokinine level. Vitrification is apparent as a poor physiological state, when tissues fade and become transparent. Especially shoots of woody species are characterized by extremely short internodiums and thickened deformed leaves without cuticula. High production of ethylene, which is known to be a growth inhibitor, can even kill the plantlets growing in a restricted space of a flask. Any agar substitutes, the modified polysaccharides of commercial marques Gerlite, Gellan, Phytagel, mostly as well conduce to vitrification (Kyte, Kleyn 1999). A number of authors mainly from developing countries (e.g. Babbar, Jain 1998; Naik, Sarkar 2001; Mohan et al. 2004, etc.) are looking for various low-cost agar substitutes for micropropagation of e.g. sugar cane, banana, apple or potato. Various organic substances (Isubgol, Tapioca, Sago, China grass, Natugel, Guar gum, sugar cane bagasse, coconut, and cotton or muslin fibre) and also some synthetic materials (nylon and polyester fibre, polystyrene foam) were tested. Nevertheless, these alternatives are also uncertain in quality and they may be chemically unstable in hot acid solutions.

Problems with micropropagation of *Pinguicula bohemica* Kraj. (Czech butterwort) have been described (Studnička 1989). This endangered herbaceous species is very responsive to ecological conditions and it manifests itself as well in natural conditions as *in vitro*. It does not grow on any media containing agar. If ½ MS medium was used, the optimal pH was 4.8–4.9. The medium solidified by agar lost its consistency and the established acidity after 20 minutes of autoclaving. Very difficult diffusion of

abscisic acid, an inhibitor produced by old leaves of plantlets, probably also presented a negative influence in agar medium. Another limiting factor playing a role in cultures of *P. bohemica* was overly restricted surface contact between the plantlets and the plus minus solid agar medium. That was the reason why a support by bath-shaped paper set in a liquid medium was also unsuitable. Sand was found to be the only beneficial support in the described special case, being chemically inert, adequately solid and perfect for diffusion of soluble substances. White silica sand marked PR 21 from the sandpit Provodín near Česká Lípa (http://www.pisky.cz) was used in laboratories of the North-Bohemian Museum in Liberec and later also of the Botanic Gardens in Liberec.

I consider the following properties as the principal advantages of the described solution:

1. It is very easy and safe to pour sand and cold liquid into flasks. The laboratory preparation is better from this point of view in comparison with risky manipulation with hot viscid agar solution.
2. A sloping wet surface for plantlets may be easily created within a flask by means of shaking with the mixture of sand and a liquid medium.
3. Plantlets can sink into sand partly and contact with medium is ideal.
4. Acidity stays at the same pH after autoclaving and no poisonous sulphuric ions occur in the flask.
5. Sand cannot absorb or adsorb any organic substances. Toxic or inhibiting products are washed from plantlets, if the flask content is briefly shaken.
6. It is very easy to remove sand from flasks after culture is finished.
7. Sand is a cheap material and is delivered in a defined quality.

These advantages were the base for my research into how to apply a similar method in *Sorbus sudetica* Tausch (Prknová 2004). It should be emphasized that woody species are more difficult than herbs in cultures *in vitro*, and conifers are especially more difficult than deciduous wood species (Kyte, Kleyn 1999). Selected results gained using *Sorbus* are briefly presented here, because research of another woody species should be derived from this experience and used in my future thesis.

MATERIAL AND METHODS

Three specimens used as a resource of seeds are cultivated in Botanic Gardens of Liberec. They were transferred to the Gardens from a Genetical Bank of the Krkonoše National Park as seedlings in 1994. From genetic viewpoint, the species is a tetraploid ($2n$ = 68) known as an apomictic wood. Its very restricted endemic population is bound to very special habitats in Krkonoše Mts. (Giant Mountains) in Bohemia (Kociánová, Štursová 1986; Kovanda 2000). We can conclude from these facts that all material useful to micropropagation should be considered as a sole mother clone.

Comparing cultures in sand and in agar

A medium of ½ RM-1964 with a content of 0.4 mg BAP, pH 5.8, was manufactured. This medium was used in part as liquid with sand (25 cm^3 sand + 20 ml solution), in part reinforced by heating 6g of agar in a 1 l medium. Cultures were incubated for 6 weeks with a daily lighting period of 14 hours. After completion of the cultures, for orientation, pH was established 3 times for each series with liquid media and with agar (by dripping liquid pH indicator for aquaristics).

Each culture was evaluated with the help of a multiplication coefficient, established as the number of viable segments acquired from the original during the incubation period. Contaminated cultures were not counted. Data were statistics evaluated by testing a zero hypotheses for the conformity of selection averages for liquid and reinforced media (*t*-test from Excel computer program tools).

Findings of the pH impact of nutrient media

A medium of ½ RM-1964 was prepared with organic substances like in the previous case, containing 0.4 mg BAP/l. The bearer was sand (25 cm^3 sand + 20 ml medium). The finished medium was divided into 3 parts and dripped at each 1N NaOH or 1N HCl set for a different acidity: pH 6.6; pH 5.6; pH 4.5 (established by colour indicator Serra-test for aquaristics). Incubation was conducted by the method noted in the previous case.

An evaluation was also conducted according to the multiplication coefficient. With the help of a *t*-test, first the zero hypothesis was tested – that the average for cultures with pH increased against the standard pH of 5.6 does not differ from the average for cultures with a pH of 5.6. By the same method, the zero hypothesis was tested for conformity of averages for pH 5.6 and pH 4.5.

RESULTS

Comparing cultures in sand and in agar

After the completion of cultivation, it was determined that when using sand, the starting pH of 5.8 remained essentially unchanged (final pH 5.6). Media reinforced by agar, however, were acidified so

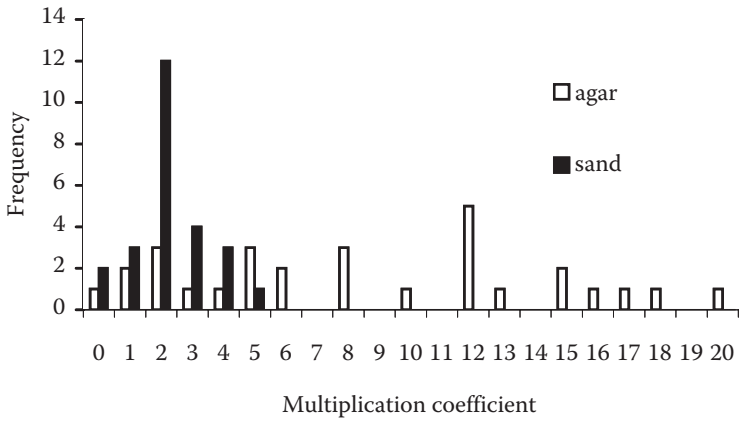

Fig. 1. Propagation of *Sorbus sudetica* in a medium with agar and or with sand, expressed by means of a multiplication coefficient

much that a pH of 4.5 was achieved or exceeded to the edge of measurement. At the same time, it was observed, that after autoclaving agar in flasks was at various degrees of liquidity. This was apparently interrupted by hydrolysis.

Methods for propagation were compared statistically and an evidential difference was determined among the mean values of the multiplication coefficient. The diffusion of values also significantly differs (Fig. 1). In agar reinforced media it is possible to achieve very good growth. Overall, their utility for experiments also for practical production purposes is burdened by the fact that they are undefined or only partially defined. This observation from the literature (Kyte, Kleyn 1999) was also confirmed during the experiment. In the case of the species *Sorbus sudetica,* growth could be favourably influenced by the actual determination of the acidic environment during the hydrolysis of agar. The following experiment originated from these possibilities and conjectures.

Determining the impact of pH on nutrient media

pH 4.5, total of 45 flasks: 1 × 7 specimens, 3 × 6 specimens, 5 × 5 specimens, 14 × 4 specimens, 14 × 3 specimens, 5 × 2 specimens, 2 × 1 specimens, 1 × 0 specimen (i.e. non-growth + apparent senescence) – average 3.56.

pH 5.6, total of 33 flasks: 1 × 5 specimens, 4 × 4 specimens, 6 × 3 specimens, 12 × 2 specimens, 6 × 1 specimen, 4 × 0 – average 2.09.

pH 6.6, total of 35 flasks: 1 × 5 specimens, 3 × 3 specimens, 11 × 2 specimens, 14 × 1 specimen, 6 × 0 – average 1.43.

The graphic expression of results of statistical evaluation, including reliability intervals, apparently demonstrates the most acidic environment as the most beneficial (Fig. 2). A medium with pH 4.5, however, due to the hydrolysis adjustment, cannot reinforce agar and the use of sand becomes necessary.

Any statistical judges cannot bear more witness in the matter of *Sorbus sudetica* qualities, because it is a species with a very homogenous population with extremely low genetic diversity. So results of experiments are adequate to the entire species.

DISCUSSION

In natural conditions *Sorbus sudetica* is no obligatory acidophyte (Kociánová, Štursová 1986). Contrary to this, under artificial conditions *in vitro*,

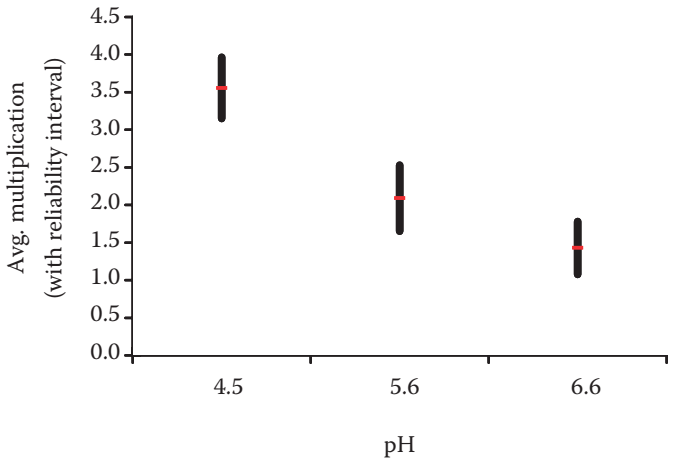

Fig. 2. Propagation of *Sorbus sudetica in vitro* using media differing in acidity

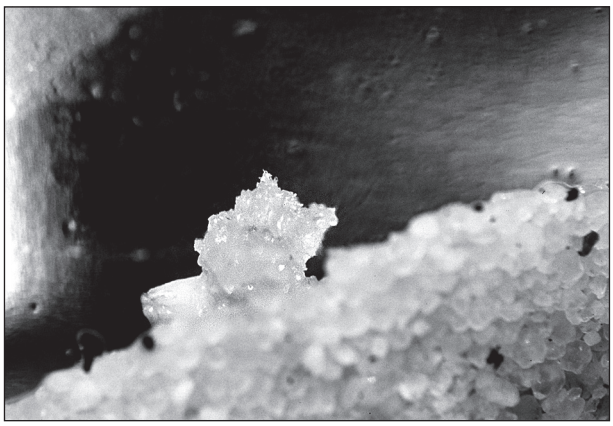

Fig. 3. Successful culture of embryogenic tissue of *Abies alba* in an acid SH medium plus silica sand

according to the presented results an acidic medium is very favourable. Lower pH in comparison with original formula of culture medium was successfully examined by other authors also in *Rhododendrons* etc. (Kyte, Kleyn 1999).

Paradoxically, in agar media it grows best if there is manifest attenuation and acidification as a result of hydrolysis of the agar. Overall, the degree of hydrolysis of agar and the characteristics of the media after sterilization in an autoclave cannot be anticipated. Microbiologists state directly that agar media with the pH of 4.5 needed for *Sorbus sudetica* cannot be completely sterilized in an autoclave (Kaprálek et al. 1967). Logically, we have come to the conclusion that chemically stable siliceous sand with numerous advantages could be an aid to resolving some difficulties with cultures of woody species *in vitro*. Initial experiments, though incomplete to publication, are indicating this possibility (Fig. 3).

Silica sand is applicable to future experiments with all wood species as an alternative for agar. New possibilities open by experiments with silica sand plus liquid mediums with low pH have a use to the laboratory Truba Breeding Station of the Forestry Research Institute in Kostelec nad Černými lesy. Low pH and high levels of phytohormones can bear interesting results by cultures *in vitro*, especially in somatic embryogenesis of *Abies* hybrids and *Ulmus glabra*. I suppose even improvement of the necessary dessication of somatic embrya if slightly wet silica sand will be replaced for paper used by authors up to now. Organogenesis of *Prunus avium* is also a job of the mentioned laboratory. The described advantages will help also in this species probably.

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Použití křemičitého písku při mikropropagaci dřevin

ABSTRAKT: Kultury *in vitro* za použití agaru jsou poněkud nejisté, protože pevnost gelu se různí podle složení média i zdroje a kvality agaru. Každé zpevňovací činidlo (např. agar) by mělo být dostatečně pevné na to, aby rostlinky udrželo, ale dostatečně tekuté, aby dovolovalo přístup k živinám a umožňovalo pronikání odpadních produktů od rostlin. Mělo by také být chemicky inertní. Agar je v těchto vlastnostech nedefinovanou složkou kultivačních médií,

obzvláště v kyselých roztocích. Křemičitý písek, dosud používaný při kulturách bylin, je vhodný i pro kultury *Sorbus sudetica*. Potřebné kyselé médium je přesně definované, pokud je agar nahrazen pískem. Podobné kultury dřevin (včetně jehličnanů) jsou předmětem dalšího výzkumu.

Klíčová slova: mikropropagace; agar; křemičitý písek; kyselé médium; *Sorbus sudetica*

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