

Effect of gelling agents on *in vitro* development of *Amelanchier canadensis* ‘Rainbow Pillar’

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Summary: *In vitro* shoot multiplication responses of *Amelanchier canadensis* ‘Rainbow Pillar’ were studied on media solidified with different gelling agents. The media were gelled either with 6.8 g l⁻¹ fibrous agar-agar, or 50.0 g l⁻¹ wheat starch, or 20.0 g l⁻¹ Guar gum, or 15 g l⁻¹ Isubgol or 50.0 g l⁻¹ wheat starch mixed with 0.5 g l⁻¹ Phytigel. Shoot cultures were grown for two months, thereafter the multiplication rates (number of newly developed shoots per explant) were counted and the length of shoots were measured. We found that the highest shoot multiplication of *Amelanchier canadensis* ‘Rainbow Pillar’ occurred on media gelled with Guar gum, while the longest shoots developed on media with Starch. About four-fold shoot number were obtained on media with Guar gum compared to the weakest results found on media gelled with Isubgol. Finally, considering all factors (shoot growth parameters, costs) the most economical gelling agent for *Amelanchier canadensis* ‘Rainbow Pillar’ was proved to be wheat starch among the tested alternatives which allows a 75.6% cost reduction.

Key words: Canadian serviceberry, gelrite, guar gum, Isubgol, psyllium, starch

Introduction

Production of pathogen-free woody propagation material with required genetic properties has worldwide been based on the micropropagation (Kozai & Kubota, 2001). The price of *in vitro* propagated plantlets – especially woody plants – is relatively high due to the specific technology elements. Each attempt, which results in increased propagation rate, greater rate of high quality plants or decreased loss during acclimatization can reduce the cost of *in vitro* propagated plantlets (Kozai & Kubota, 2001). Although liquid medium can be used effectively in automated micropropagation systems (Krueger et al., 1991; Robacker & Simonton, 1992), the solidification of medium seemed to be necessary in traditional systems, because using of liquid medium resulted in hyperhydric shoots in *Amelanchier x grandiflora* Rehd. ‘Princess Diana’ when cultures were continuously in contact with medium (Krueger et al., 1991).

Because of its stability, high clarity, non-toxicity and metabolic inactivity the agar extracted from red algae has been the most frequently used for gelling media (McLachlan, 1985). Even though the cost of medium preparation constitute a small part of the total cost (George, 1996), components of the medium is quite expensive to obtain, especially the plant tissue culture tested agar-agar, which has commonly used for woody plants (Kumar Goel et al. 2007). Alternatives including Isubgol (the husk derived from the seeds of *Plantago ovata*), Guar

gum (galactomannan derived from the endosperms of *Cyamopsis tetragonoloba*), several type of starches (wheat, cassava, corn, potato, sago etc.) and other additives (bagasse, Xyloglucan extracted from seeds of *Hymenaea courbaril* etc.) have been studied in several experiments to find a cheaper solution for gelling media (Babbar and Jain, 1998; Naik and Sarkar, 2001; Lima-Nishimura et al., 2003; Mohan et al., 2004; Babbar et al., 2005).

Several blends of agar and its cheap alternatives (guar gum, isubgol and xanthan gum) were evaluated, and none of them had rheological properties comparable to agar gelled medium. However, responses of *Albizia lebeck* tissue culture to majority of blends were the same or even better than the control (agar) (Jain-Raina & Babbar, 2011). Each types of gelling agents (Plant Agar, Gelcarin GP-812, Guar gum, Isubgol) was effective in micropropagation of blackberry (*Rubus laciniatus* ‘Thornless evergreen’), however the highest multiplication rate was achieved on medium gelled with Plant Agar, while plantlets grown on medium solidified by Guar gum were extremely vigorous and well developed (Fira et al., 2010). Blends of agar and guar increased the number of regenerated shoots in micropropagation of pear (*Pyrus communis* L., ‘Durondeau’) compared to media solidified by agar (Lucyszyn et al., 2006).

Mixtures of agar and Isubgol also were effective in *in vitro* regeneration of potato and *Stevia* provided that the rate of Isubgol in the mixture does not exceed 50% (Khan et al., 2012).

Daud et al. (2011) also tested some starch sources (cassava, rice and corn flour and potato starch) as gelling agents compared to Oxoid technical agar. They found that 60 g l⁻¹ cassava flour added to the MS (Murashige and Skoog, 1962) medium resulted in the highest number of regenerated shoots in *Celosia* sp. Blends of corn or potato starch and agar were also highly effective in potato micropropagation (Mohamed et al., 2010).

The rate of shoot growth during establishment of *in vitro* shoot culture of walnut was also influenced by the gelling agents; agar, cassava flour and tapioca pearls were added to the initiation media. Albeit significant differences in shoot growth of cultivars ('Mihaela', 'Muscelean' and 'Jupâne ti') were observed, each genotype preferred tapioca pearls as gelling agent (Gotea et al., 2012). Kuria et al. (2008) studied the effect of gelled (agar, cassava starch and their mixture) and liquid media on the fresh weight, number of nodes and survival of micropropagated potato plantlets. They found, that the number of nodes was significantly the lowest on media gelled with agar and the fresh weight was the highest on liquid media, while the survival percentage was higher on gelled media. All parameters considered, media gelled with 10% cassava starch were proven to be the most effective.

Phytigel enhanced the *in vitro* tuberization of potato (Arregui et al., 2003) and adventitious bud development in pear (Chevreau et al., 1997), increased the rate of survival during establishment of *in vitro* culture of *Populus trichocarpa* (Kang et al., 2009) applied either alone or in combination with agar was also effective in shoot multiplication of *Maranta leuconeura* (Ebrahim & Ibrahim, 2000).

In our experiments shoot multiplication responses of *Amelanchier canadensis* 'Rainbow Pillar' were studied on media gelled with fibrous agar-agar (control), wheat starch, Guar gum, Isubgol and blend of wheat starch and Phytigel.

Materials and methods

Multiplication of *in vitro* shoots of *Amelanchier canadensis* 'Rainbow Pillar' was made on medium containing MS salts (Murashige and Skoog, 1962) and MS vitamins except that vitamin B₁ was added to the medium at 1.0 mg l⁻¹ instead of 0.1 mg l⁻¹. The medium was also supplemented with 100 mg l⁻¹ myo-inositol, 3% sucrose, 6.0 g l⁻¹ agar-agar and 0.5 mg l⁻¹ 6-benzyladenine (BA). The same medium was used for gelling agent experiments, in which media were gelled with either 6.8 g l⁻¹ fibrous agar, or 50.0 g l⁻¹ wheat starch, or 20.0 g l⁻¹ Guar gum, or 15 g l⁻¹ Isubgol or a blend of 50.0 g l⁻¹ wheat starch and with 0.5 g l⁻¹ Phytigel. The media were sterilised by ST-124/2 autoclave for 30 min. at 121 °C and 10⁵ Pa after adjustment of pH to 5.8.

Shoot-tips in 2.0 cm length were used as explants. Four explants were placed per jar (750 ml Kilner jar) containing 100

ml medium. Shoot cultures were grown for two months in a growing room at 22°C, 16 h photoperiod and at a PPF of 105 µmol s⁻¹ m⁻². Warm-white lamps (Tungsram F74) were used for illumination of cultures. Each treatment included ten replicates (i.e. 40 explants in total were observed) and experiments were repeated twice in time. After two months the multiplication rates (number of newly developed shoots per explant) were counted and the length of shoots were measured. The data were analysed by ANOVA and Tukey's test was applied as post-hoc test using the SPSS 13.0 for Windows software package.

Results

Different gelling agents resulted in significant differences in the multiplication rates of *Amelanchier canadensis* 'Rainbow Pillar' (Figure 1). The most shoots (154.8 per explant) developed on media gelled with Guar gum. Similarly, the multiplication rates were also very high on media contained agar and wheat starch (111.2 and 110.2 new shoots per explant, respectively), while applying Isubgol and Starch+Phytigel mix significantly inhibited the rate of shoot proliferation (34.9 and 65.9 new shoots per explant, respectively) compared to the media with Guar gum. Even when present in small quantities (0.5 g l⁻¹) the presence of Phytigel inhibited the shoot multiplication of *Amelanchier canadensis* 'Rainbow Pillar': almost only half of the shoots developed on media containing Starch+ Phytigel (65.9) as gelling agents compared to the media gelled with only starch (110.2). Considering only the usable shoots (larger than 20 mm) the best propagation rate also found on the media gelled with Guar gum.

The smallest shoots (24.0 mm) developed on media containing Isubgol, while the longest shoots grown on media gelled with starch and the mixture of starch and Phytigel (31.5 and 30.8 mm, respectively) (Figure 2). Except for Isubgol, each alternative gelling agent increased significantly the length of shoots compared to the shoot length measured on medium with agar. However, the lengths of newly developed shoots were adequate in each treatment: shoots larger than 20 mm are suitable for subculture and also for rooting and *ex vitro* acclimatization.

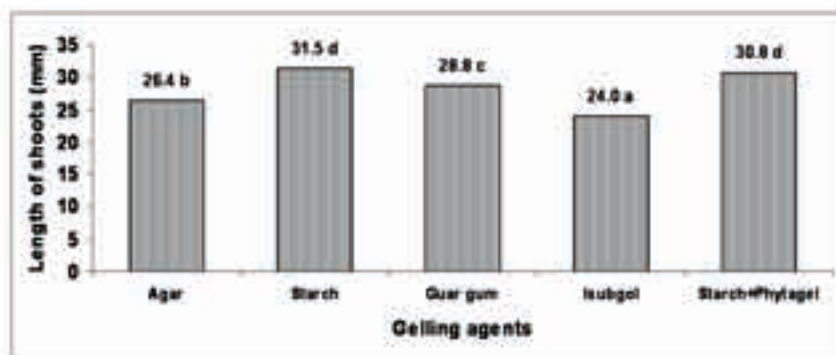


Figure 1. The total numbers of newly developed shoots and numbers of new shoots, longer than 20 mm on media gelled by different gelling agents. Significant differences ($P \leq 0.05$) between the media gelled by different gelling agents are indicated by different letters

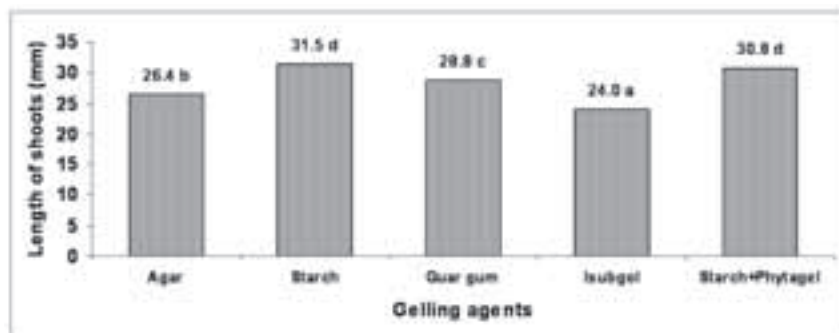


Figure 2. The effect of the gelling agents on the shoot length (mm). Significant differences ($P \leq 0.05$) between the media gelled by different gelling agents are indicated by different letters.

Discussion

Besides cost reduction adequate hardness of the medium is of importance during plant tissue cultures. If media are too soft the hyperhydricity can be frequent in *in vitro* cultures while too hard media could result in inhibited growth (Gangopadhyay et al., 2009). In our experiments the physiological states of shoots were adequate for each treatment; no hyperhydrated culture or other physiological disorders were observed, however significant differences were observed in both parameters observed on media gelled differently (Figure 3).

Each tested morphogenic response (seed germination, shoot proliferation, rooting, androgenesis, somatic embryogenesis) was enhanced in *in vitro* cultures of several plant species (*Linum usitatissimum*, *Brassica juncea*, *Crataeva nurvala*, *Nicotiana tabacum*, *Calliandra tweedii*) on media solidified by Guar gum (Babbar et al., 2005). We also observed that the multiplication of shoot cultures of *Amelanchier canadensis* 'Rainbow Pillar' were the best on media gelled with Guar gum, while the longest shoots developed on media with Starch. Stimulating effect of Guar gum was spectacular: more than four-fold shoots were obtained compared to the weakest results found on media gelled with Isubgol. However, the propagation rates found on media gelled with Starch and Guar gum did not differ significantly compared with one another and with the control medium (agar).

One of promising alternatives was Isubgol, because of its low price (Table 1). Babbar and Jain (1998) applying Isubgol obtained that results comparable to agar media in *in vitro* cultures of *Syzygium cuminii* and *Datura innoxia*. In adventitious regeneration experiments with woad (*Isatis tictoria*) Saglam and Cifici (2010) found more regenerated shoots on media supplemented with Isubgol at 15 g l⁻¹

concentration compared to media gelling with agar. Khan et al. (2012) using blends of agar and Isubgol reduced the cost of plant tissue culture medium solidification by 47.5%. However, in our experiments the Isubgol was the least efficient alternative gelling agent resulting in the lowest propagation rate and the smallest shoots of *Amelanchier*.

Blends of agar and other gelling agents can be effective and cost-saving possibilities (Mohamed et al., 2010; Dobránszki et al., 2011). Ebrahim and Ibrahim (2000) observed



Figure 3. *In vitro* shoots of *Amelanchier canadensis* 'Rainbow Pillar' developed on media gelled with either 6.8 g l⁻¹ fibrous agar-agar (A); or 20.0 g l⁻¹ Guar gum (B); or 50.0 g l⁻¹ wheat starch (C); or a blend of 50.0 g l⁻¹ wheat starch and with 0.5 g l⁻¹ Phytigel (D); or 15 g l⁻¹ Isubgol (E).

Table 1. Comparison of the costs of media gelled with different gelling agents. The gelling agents, used in our experiments marked by *.

Gelling agents	Cost per kg €	Conc. in medium (g/l)	Cost per liter €
Wheat starch * (commercial)	1.78 €	50	0.089
Wheat starch (Sigma)	23.90 €	50	1.195
Agar-agar * fibrous	54.05 €	6.8	0.368
PTCT ¹ Agar (Sigma)	593.38 €	5.6	3.323
PTCT ¹ Agar (Oxoid)	124.07 €	5.6	0.695
Guar gum * (Sigma)	110.44 €	20	2.209
Phytigel (Sigma)	413.82 €	2.5	1.035
Isubgol *	15.61 €	15 g/l	0.234

¹ PTCT = plant tissue culture tested

that combination of agar and Gelrite (3+1 g l⁻¹) resulted in the best development and growth of *Maranta leuconeura* 'Kerchoviana' shoots, but a combination of agar and Gelrite with higher rate of agar (5+0.5 g l⁻¹) was superior considering the rooting response of shoots. However, we found in present study that the blend of starch and Phytigel resulted in low number of new shoots similarly to the Isubgol. The presence of Phytigel inhibited only the shoot multiplication, while the mean shoot length was the same on media containing starch alone or combined with Phytigel. Ivanova et al. (2007) also observed that *in vitro* shoot cultures of *Aloe polyphylla* did not respond well to Gelrite; the propagation rate was lower and the hyperhydrated cultures were more frequent on medium gelled with Gelrite compared to medium containing agar. When the concentration was increased from 2.4 up to 16 g l⁻¹ the rate of hyperhydricity was reduced but the number of shoot also decreased (Ivanova and Van Staden, 2011).

Although the highest number of new shoots was achieved on medium containing Guar gum, applying the wheat starch for gelling medium was proved to be the most effective for *Amelanchier canadensis* 'Rainbow Pillar'; the propagation rate was similar to the control (agar) medium, and the shoots were the longest. Moreover, the cost of wheat starch is the best among tested alternatives: the price of commercial wheat starch is a very small proportion of the price of agar, even the pure wheat starch (Sigma) price is only half of the price of agar (Table 1). Cassava starch was found to be the most effective for *Celosia* sp. (Daud et al., 2011) and Kuria et al. (2008) also used it for potato propagation at 10% in medium and reduced cost by 42.5% compared to medium gelled by agar. Corn and potato starch were also usable gelling agents in combination with Gelrite in *Musa* 'Grande Naine' shoot tip culture, moreover 90% cost reduction was achieved when sucrose source was also replaced by commercial sugars (Kodym and Zapata-Arias, 2001).

Table 2. Comparison of the economy of media solidified with different gelling agents

Gelling agents	Cost per liter €	Yield*	Cost of gelling per new shoots x 10 ⁻⁴ €	Changes in the cost of gelling (%)
Wheat starch* (commercial)	0.089	4408	0.2019	-75.6%
Agar-agar * fibrous	0.368	4448	0.827	0
Guar gum * (Sigma)	2.209	6192	3.57	+331%
Wheat starch * (commercial) + Phytigel	0.296	2636	1.123	+35.8%
Isubgol *	0.234	1396	1.68	+103.1%

* The number of newly developed shoots using I I medium (counting from propagation rates, which can be realised on the medium in the case of four explants on 100 ml medium)

The cost of Guar gum is relatively high (Table 1) and when added to the media alone, at least 20 g l⁻¹ was necessary to obtain medium with appropriate consistency. In this case the cost of medium is high. However, Dobránszki et al. (2011)

used blend of Guar gum and agar for micropropagation of apple and black locust effectively, about 42% cost reduction could be achieved.

Considering all factors examined, such as propagation rates, shoot lengths, media costs, cost per plantlets, the most effective gelling agent for *Amelanchier canadensis* 'Rainbow Pillar' is the wheat starch among the tested alternatives. Although a very cheap agar-agar (fibrous) was selected as control, which in itself was a cost reduction, we could reach further cost reduction applying wheat starch (75.6% cost reduction could be reached considering only the gelling of the medium) (Table 2).

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