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# Large scale production of *in vitro* plantlets of *Stevia rebaudiana* (Bertoni) using low-cost gelling agents as alternative to commonly used agar

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## Abstract

Plant cell culture is an advance technology for large scale production of medicinal plants of commercial importance but high cost of the media is one of the major issues for economical growers. One of the most expensive agent of culture media is agar and therefore, the main objective of this study was to select best agar alternative among isubgol, semolina, laundry starch, sago, and corn starch. These alternatives were tested during the morphogenesis of *Stevia rebaudiana* (Bert.). *S. rebaudiana* (Bert.) is one the famous sweet herb that produce sweet diterpenes that could be used as sugar alternative for diabetic patients due to its non-toxic nature. Here, the synergistic combinations of isubgol and Agar+isubgol augmented Murashige and Skoog (MS) media supplemented with 2.0 mg/L benzyl aminopurine (BAP) and 0.5 mg/L gibberellic acid ($GA_3$) was found to be effective for shoot induction (100%) and shoot morphogenesis than control (MS media without plant growth regulators). The MS media supplemented with 0.5 mg/L naphthalene acetic acid (NAA) along with corn starch (90 g/L) is the best combination for rapid root initiation, however, corn starch/sago alone or the synergistic combination of with agar or sago significantly improved root organogenesis (100%) in medicinally important *Stevia rebaudiana* (Bert.). However, sago encourage mean shoot and root length and reduced 90% cost than agar. Semolina was the cheapest agar substitute by reducing the cost up to 97.75% and could be scale up for higher production of plantlets of commercial importance.

**Keywords:** *Stevia rebaudiana*, Morphogenesis, Gelling agents, Isubgol, Sago, Semolina, Corn starch, BAP

## 1. Introduction

*Stevia rebaudiana*, a perennial herb, belonging to Asteraceae family is one of the commercial and economically important plant [1]. It is a medicinal plant and sweet in taste because of the presence of some valuable compounds (Steviol glycoside) in its leaves [3-5]. As a crop, this plant has a major potential to produce a high potent natural sweetener [6]. Steviol glycosides are generally considered sweeter (300-400 times) than commercially produced sugar [7, 8]. It is safe for diabetic patients in keeping blood sugar at safe level. It also accelerates the release of insulin and stabilises blood glucose level [1, 6]. Due to its low caloric property, many European, American and Asian countries have permitted the use of stevia extract as sweetener for different commodities [8, 6]. In many developed countries, different stevia-based products are being launched such as jams, candies, drinks, and ice cream etc. [1-4, 8, 6].

The stevia plants are found worldwide and mostly distributed in Brazil, Argentina, Mexico, USA with 500-3,500 m altitudes from sea level. This genus normally grows in semi-dry mountainous terrains and habitats such

as scrub forests, mountain slopes, grasslands, conifer forests and in sub-alpine vegetation [9]. However, stevia is the native species of Paraguay (Rio-Monday) and later on the Italian-Swiss scientist Dr. M. S. Bertoni collected it from Paraguayan Indians and Mestizos and isolated the sweet diterpenes (steviosides) from the leaves [10]. The stevia plant is small shrub that is perennial in nature with well organizes stem (mostly annual and sublinguious) and rooting system (perennial, fibrous and filiform) with maximum height ranges from 65 to 80 cm. The leaves, branches and stem are covered by fine hairs and the arrangement of the leaves is alternate around the stem. The leaves are lanceolate in nature and the internode size vary and reached from 2 to 4 cm long [9]. The self-incompatibility, infertility, inadequate pollination desirability hinders the natural growth of the plants and also interrupt the breeding due to the heterozygous nature [9].

Propagation of stevia can be done by sexual (seed) as well as asexual means (vegetative cuttings) [3, 4]. However, asexual means of propagation require proper environmental conditions and favorable season, huge amount of planting material, more labor as well as more space [2, 8]. Self-incompatibility and poor seed viability are also one of the limiting factors for sexual means of propagation [1, 3]. Propagation through plant tissue culture is one of the solutions to overcome all the problems associated with conventional approaches and to meet the increasing demand of this plant. This method ensures propagation of mass population of disease-free plants in a limited time without any seasonal limitations [3-5]. Organogenesis of shoots and roots in micropropagation produce plants large in number, homogeneous in nature and can be obtained from a small explant in a short period of time without any seasonal limitations [8].

However, an often-mentioned drawback of plant tissue culture technology is the higher cost involved in comparison with conventional methods of propagation. The high cost of the media is because of the use of expensive media components such as growth regulators, organic and inorganic supplements, carbon sources and gelling agents in culture media [11]. Generally, in micropropagation *in vitro* shoots require support. Traditionally agar has been widely used as a gelling agent for this purpose due to its clarity, resistance to metabolism and nontoxic nature. Commercially, it is obtained from some specific sea weeds (spp. *Gelidium, Gracillaria* and *Pterocladia*) [12]. This hydrocolloid is composed of agarobiose (3-β-Dgalactopyranosyl-(1, 4)-3, 6-anhydro-α-L-galactose). However, 70% of the media cost is contributed towards agar alone. It is therefore important to find some alternative to agar for the reduction of overall media cost. The replacement of agar with some inexpensive agar substitutes either completely or somehow partially could be a useful strategy [11, 12].

Botanical starches such as sago is obtained after processing of raw material produced by ago palm (*Rumphii, sagu spp.* Genera; Metroxylon) [12]. Similarly, corn starch and laundry starches are major carbohydrate sources. Starch granules contain two large size molecular components i.e. amylose and amylopectin. Amylose has a property of producing strong films and tougher gels. In comparison, amylopectin produces soft gels and weak films but is more stable when dispersed in water. Semolina is a wheat middling of different wheat species (durum wheat), coarse in texture but is purified in nature. Moreover, gelrite is another gelling alternative and paly a key role in root stimulation under in vitro conditions [13-14]. Isubgol, a polysaccharide (colloidal mucilage), used as a laxative, produced in abundance from the husk of *Plantago ovata* Forsk [13].

This study was conducted to find dependable, cheaper, easily available replacement of agar, which could be used in culture medium for micropropagation of *Stevia rebaudiana* (Bert.), to produce large number of plantlets commercially with low cost of production. Therefore, in this experiment, a variety of agar alternatives was used for the micropropagation of stevia in order to compared its cost with commonly used agar in MS media and to study the different parameters such shoot and root morphogenesis in response to agar and agar alternatives.

## 2. Materials and methods

### *2.1 Explant collection and sterilization*

*Stevia rebaudiana* (Bert.) seeds of Paraguay variety were procured and successfully cultivated through *in vitro* techniques at plant tissue culture Lab., PCSIR Laboratories Complex Peshawar during the year 2009-2010. After several trials the stevia plants were raised and multiplied followed by successful acclimatization at experimental Garden, PCSIR Laboratories Complex Peshawar-Pakistan. These stevia plants were brought to Garden Nursery, Department of Horticulture, the University of Agriculture Peshawar. Newly sprouted leaves of *Stevia rebaudiana* were collected from plants available in the field and were thoroughly washed with tap water to remove dust particles in April, 2021.

### *2.2 Explant surface disinfection*

The collected leaves were subsequently dipped in autoclaved distilled water in sterilized jars. Before inoculation of explant to culture media. The leaf explants were initially washed with local detergent for 10 min to remove surface particles. Step by step, the leaf explants were surface disinfected by subjecting these leaves for 90 seconds in 0.2% mercuric chloride ($HgCl_2$) solution. After exposure to $HgCl_2$, the explants were gently rinsed with sterile distilled water to avoid cell death. The surface disinfected explants were placed on autoclaved filter

paper to remove water droplets and $HgCl_2$. Sterilized surgical blade was used for cutting of leaf explants to obtain 3-4 $cm^2$ pieces for the subsequent experiments.

*2.3 Growth conditions*

In this study, MS medium (Murashige and Skoog) [14] solid media was used for cultures establishment. The MS media was fortified with 7.5 g/L agar for solidification to support the leaf explants and shoot and root development. The solid media was augmented with 3 % sucrose as one of the limiting substrates and carbon energy source. The MS-solid media without plant growth regulators (PGRs) was used as control. The pH of the solid media was adjusted with weak acid/base ranges from 5.6 to 5.8 to get the desirable results. Further, the media was autoclaved at 121°C for 20 min to remove any possible contamination. The surface disinfected explants were cultured onto solid MS-media and placed in growth room with normal temperature of 25 °C ± 2.5, photoperiod (16/8 h) and the intensity of light was kept as 40-mol/$m^2$/s using lux meter.

*2.4 Micropropagation*

Uniform size (3-4 $cm^2$) of sterilized leaves were cultured on Murashige and Skoog (MS) medium [12] having carbohydrate (sucrose) at the rate of 30 g $L^{-1}$ and optimized level of plant growth regulators (PGRs i.e. BAP; 2 mg/L and 0.5 mg/L $GA_3$) from already conducted experiment of Aman et al. [2] on direct shoot organogenesis of stevia. For root induction, developed plantlets were sub-cultured in MS media (half strength) having 0.5 mg/L Naphthalene acetic acid (NAA). Growth media for both shooting and rooting were solidified with different gelling agents, used alone as well as in combination (1:1) with agar. pH was adjusted prior the addition of gelling agents from 5.6 - 6.0. Agar gelled media was kept as control.

Culture medium is usually augmented with gelling agents to increase its thickness so that tissues and organs can remain above the surface. Amount of different gelling agents to be added, were finalized by reviewing literature and by making some preliminary investigations (Figure 1).

Important factor for media solidification is that it should not be too soft or too hard because in both cases growth of roots and shoots is affected. Because physical consistency of culture medium strongly influences the growth of plantlets. The detail of the low-cost gelling agents alone and in combination is given below in Table 1.

**Table 1** Multiple alternatives of agar (alone and in equal ratio with agar) for organogenesis of shoots and roots in *Stevia rebaudiana*.

| Treatments | Agar substitutes | Amount used (g/L) |
|---|---|---|
| T1 | Agar | 20.0 |
| T2 | Semolina | 90.0 |
| T3 | Sago | 110.0 |
| T4 | Laundry Starch | 90.0 |
| T5 | Corn Starch | 90.0 |
| T6 | Isubgol | 28.0 |
| T7 | Agar + Semolina | 10.0 + 45.0 |
| T8 | Agar + Sago | 10.0 + 55.0 |
| T9 | Agar + Laundry Starch | 10.0 + 45.0 |
| T10 | Agar + Corn Starch | 10.0 + 45.0 |
| T11 | Agar + Isubgol | 10.0 + 14.0 |


**Figure 1** Pictorial presentation of starch, semolina, sago and Isubgol (Adopted from internet; Wikipedia).

*2.5 Procedure for recording data on various attributes during the study*

Days to shoot and root emergence were noted by calculating difference between days of inoculation and the first day at which the shoots or roots emerged in the culture media. Average of all the replications was then made. Similarly, the number of shoots and roots induced were evaluated by totaling the shoot number/root number in jars of each individual treatment, then their mean was taken, by taking the number of jars as denominator. Size of shoot/root were obtained for each treatment by measuring the lengths of elongated shoots/roots, their mean was then taken by taking the number of jars as denominator again. Data related to root and shoot induction, percentage was analyzed by calculating the shoot/root number induced in replications of every treatment and the percentage was calculated using given formula (Equation (1)).

$$Induced\ shoots/roots\ (\%) = \frac{shoots\ induced\ per\ explant/roots\ induced\ per\ plantlet)}{\text{total (explants or plantlets)}} \times 100 \quad (1)$$

The term explant is used for shoot induction while plantlets are the already developed shoots from the explant, for further root induction.

*2.6 Statistical Analysis*

An independent replicated experiment ($n$=3) was used for shoot and root induction in *Stevia rebaudiana* (Bert.) and repeated at least twice to obtain accurate results in CRD. Each time, 9 jars per treatment were cultured with 3 explant/plantlets for shoot and root induction, respectively. To test whether the observed data and differences in it were significant or not, data were subjected to ANOVA using Statistix software (8.1) to get LSD values to find differences (probability level) among means ($p$=0.05).

## 3. Results and discussion

*3.1 The effect of agar and its substitutes on days to shoots and roots emergence*

Different gelling agents were used to investigate the shoot and root emergence and morphogenesis in medicinally *Stevia rebaudiana*. Agar the culture media was taken as control for both experiments (shoot and root morphogenesis). The earliest shoot induction (12.11 days) was observed in agar + isubgol solidified (10+14 g/L) media. Agar (20 g/L) alone took 29.8 days to obtained shooting response. Among these gelling agents, laundry starch (90 g/L) took maximum days (45.2) in shoot induction followed by corn starch (90 g/L) with 39.3 days (Table 2). In contrast, corn starch (90 g/L) was the earliest to induce roots (10.5 days). While in case of root emergence, agar took 19.4 days to show any signs of root emergence. Combination of agar and semolina (10+45 g/L) took comparatively long time (30.1 days) in root emergence. Media solidified with semolina failed to induced any shooting or rooting response (Table 2).

**Table 2** Mean average data along with standard errors (±) and least significant differences (LSD; common alphabets) representing days to shoot and root emergence in response to agar and its low-cost alternatives during micropropagation of *Stevia rebaudiana*.

| Agar substitutes | Amount Used (g/L) | Days to Shoot Induction | Days to Root Induction |
|---|---|---|---|
| Agar | 20 | 29.8 ± 2.1 d | 19.4 ± 0.66 c |
| Sago | 110 | 32.3 ± 1.9 c | 11.0 ± 0.22 ef |
| Laundry starch | 90 | 45.2 ± 1.4 a | 25.1 ± 0.32 b |
| Corn starch | 90 | 39.3 ± 2.5 b | 10.5 ± 0.11 f |
| Isubgol | 28 | 24.1 ± 0.92 f | 11.1 ± 0.12 e |
| Agar + semolina | 10+45 | 14.2 ± 0.33 h | 30.1 ± 0.76 a |
| Agar + sago | 10+55 | 21.1 ± 0.61 g | 18.9 ± 0.08 c |
| Agar + laundry starch | 10+45 | 27.2 ± 0.53 c | 11.1 ± 0.07 ef |
| Agar + corn starch | 10+45 | 29.6 ± 1.17 d | 11.2 ± 0.066 e |
| Agar + Isubgol | 10+14 | 12.1 ± 0.16 i | 12.1 ± 0.23 d |
| LSD (P≤ 0.05) | | 0.84 | 0.63 |

*3.2. Influence of agar and other gelling agents on number of shoots and roots induction*

Shoot and root organogenesis is one of the key attributes that indicate success in micropropagation. During shoot and root organogenesis, significant variations were observed in number of shoots and roots induction in different media solidified with different gelling agents alone or in combination with agar (Figure 2 and 3). Media solidified with isubgol (90 g/L) induced maximum shoots (46.3) per explant followed by media gelled with sago (110 g/L) with 92.8 number of shoots. Rest of the media gelled with agar and other alternatives to agar alone and

in combination with agar induced statistically similar number of shoots per explant (Table 3). In case of rooting response, the highest number of roots (8.3) were emerged in plantlets cultured in media solidified with agar + sago (10+55 g/L), which was statistically similar with other growth medium solidified with agar (20 g/L) alone and media gelled with agar in combination with corn starch (10.45 g/L) with 7.5 and 6.7 number of roots, respectively. However, poor results in case of root numbers (1.2) were observed in growth medium gelled with laundry starch (90 g/L) (Table 3).

**Table 3** The effect of agar and agar substitutes in culture media on number of mean number of shoot and root organogenesis of *Stevia rebaudiana.* Mean average data along with standard errors (±) and the common alphabets represents the LSD.

| Agar Substitutes | Amount Used (g/L) | Number of Shoots per Explant | Number of Roots per Shoot |
|---|---|---|---|
| Agar | 20 | 14.8 ± 0.4 c | 7.5 ± 0.45 ab |
| Sago | 110 | 29.8 ± 1.1 b | 3.6 ± 0.01 dc |
| Laundry starch | 90 | 4.9 ± 0.05 c | 1.2 ± 0.01 f |
| Corn starch | 90 | 10.5 ± 0.22 c | 4.7 ± 0.2 cde |
| Isubgol | 28 | 46.3 ± 2.3 a | 3.7 ± 0.04 de |
| Agar + semolina | 10+45 | 3.8 ± 0.08 c | 5.4 ± 0.08 bcd |
| Agar + sago | 10+55 | 3.0 ± 0.04 c | 8.3 ± 0.77 a |
| Agar + laundry starch | 10+45 | 1.6 ± 0.01 c | 6.1 ± 0.03 bc |
| Agar + corn starch | 10+45 | 3.6 ± 0.05 c | 6.7 ± 0.04 abc |
| Agar + Isubgol | 10+14 | 10.8 ± 0.66 c | 2.6 ± 0.09 ef |
| LSD (P≤ 0.05) | | 14.33 | 2.18 |

*3.3. Average length of shoots and roots in agar and other low-cost substitutes gelled media*

In the present study, agar substitutes alone and in combination had significant effect on increasing shoot and root lengths (Figure 2 and 3). Sago (90 g/L) gelled media was found the most suitable in obtaining shoots with maximum length (8.1 cm). Isubgol was second in performance and displayed 4.96 cm average shoot length. Agar gelled media (20 g/L) resulted shoots with 1.9 cm length. Poor growth of the shoots with minimum length (0.7 cm) was observed in laundry starch (90 g/L) gelled media (Table 4). Similarly, sago gelled (90 g/L) media also encouraged average root length (4.4 cm) followed by agar + sago (10+55 g/L) gelled media with average root length of 3.2 cm. While root length of isubgol gelled medium was somehow not satisfactory, because of its mucilaginous property, it didn't allow the roots to grow longer. However, medium having agar (20 g/L) resulted roots with 0.8 cm length. Roots induced in laundry starch (90 g/L) gelled media were observed with poor length (0.1 cm) (Table 4).

**Table 4** Shoot and root length of Micropropagated plantlets of *Stevia rebaudiana* in media solidified with agar and other low-cost gelling agents. Mean average data along with standard errors (±) and the common alphabets represents the LSD.

| Agar Substitutes | Amount Used (g/L) | Shoot Length (cm) | Root Length (cm) |
|---|---|---|---|
| Agar | 20 | 1.9 ± 0.01 cde | 0.8 ± 0.01 c |
| Sago | 110 | 8.1 ± 0.51 a | 4.4 ± 0.02 a |
| Laundry starch | 90 | 0.7 ± 0.01 e | 0.1 ± 0.00 e |
| Corn starch | 90 | 1.0 ± 0.00 e | 2.8 ± 0.01 b |
| Isubgol | 28 | 4.9 ± 0.04 b | 0.3 ± 0.02 cde |
| Agar + semolina | 10+45 | 2.9 ± 0.06 c | 0.2 ± 0.00 de |
| Agar + sago | 10+55 | 2.0 ± 0.3 cde | 3.2 ± 0.02 b |
| Agar + laundry starch | 10+45 | 1.5 ± 0.01 de | 0.9 ± 0.01 c |
| Agar + corn starch | 10+45 | 2.6 ± 0.01 cd | 0.8 ± 0.01 cd |
| Agar + Isubgol | 10+14 | 3.1 ± 0.01 c | 0.5 ± 0.02 cde |
| LSD (P≤ 0.05) | | 1.37 | 0.57 |

*3.4. Shoots and roots induction percentage in agar and its substitutes gelled media*

Percentage of shoot and root induction was assessed in all the media's gelled with agar or its substitutes alone or in combination with agar (Figure 2 and 3). In case of shoot induction, isubgol gelled medium was found the most promising one, induced 100 % response followed by agar gelled media (20 g/L) with 66.6% shooting response. Media solidified with laundry starch (90 g/L) resulted poor shooting (23.3%) response (Table 5). While in case of root organogenesis, agar, corn starch and sago alone as well as in combination with agar (1:1) induced 100% response. Agar and isubgol in combination (10+14 g/L) resulted 80% rooting response, which was statistically followed by agar and laundry starch (10+45 g/L) gelled media with 75% rooting response. Laundry starch added alone as solidifying agent in rooting media showed only 9.9 % rooting in *in-vitro* shoots of *Stevia rebaudiana* (Table 5).

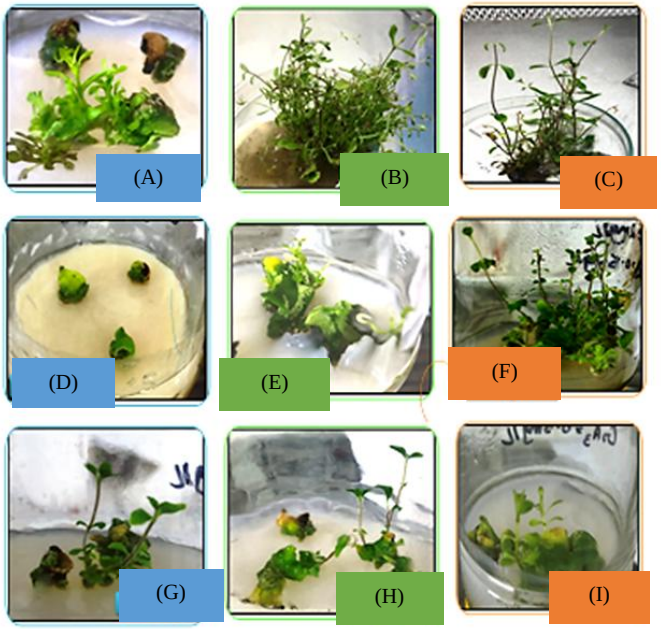


**Figure 2** In-vitro shoot morphogenesis of *Stevia rebaudiana*, explant culture on different alternatives of gelling agents + MS media + PGRs (A) leaf explant of *Stevia rebaudiana* culture on agar (B) leaf explant cultured on Isubgol, maximum number of shoots and maximum shoot percentage were observed in isubgol gelled medium (C) maximum shoot length was observed in sago gelled medium (D) explant culture on semolina (E) explant culture on media supplemented with Corn Starch (F) rapid shoot initiation from leaf explant of *Stevia rebaudiana* on media supplemented with Agar + Isubgol (G) leaf explant cultured on the medium supplemented with Agar + Sago (H) explant cultured on media supplemented with Agar + Corn Starch (I) *Stevia rebaudiana* leaf explant cultured on MS medium supplemented with Agar + semolina.

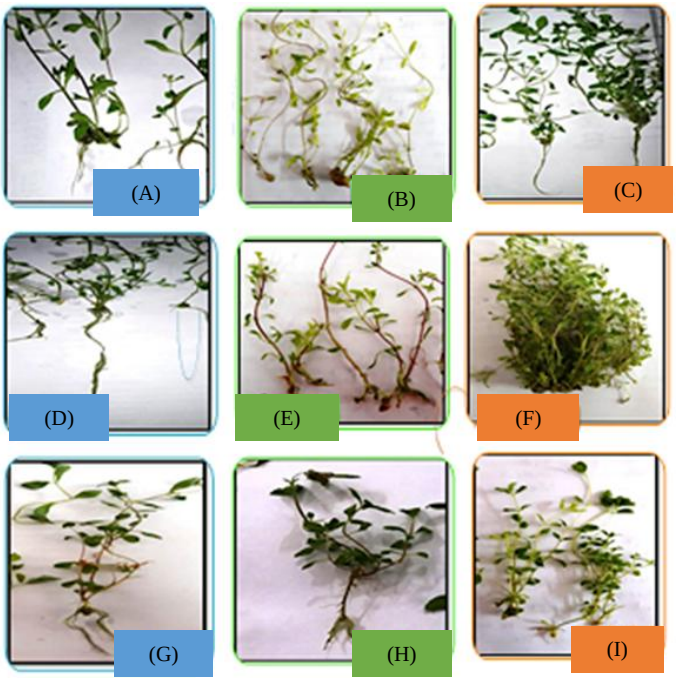


**Figure 3** In-vitro root morphogenesis of *Stevia rebaudiana* on different alternatives of gelling agents + MS media + PGRs, (A) shoots of *Stevia rebaudiana* culture on Agar (B) Shoots of *Stevia rebaudiana* culture on Isubgol (C) shoots culture on Sago, maximum root length was observed in Sago gelled medium (D) shoots cultured on media supplemented with Corn Starch, early root induction, maximum number of roots and maximum roots percentage were observed in corn starch gelled medium (E) shoots culture on semolina (F) shoots cultured on medium supplemented with Agar + Isubgol (G) shoots cultured on medium supplemented with Agar + Sago gelled media (H) shoots of *Stevia rebaudiana* culture on MS medium supplemented with Agar +Corn Starch gelled media and (I) *Stevia rebaudiana* shoots culture on MS medium supplemented with Agar + semolina gelled media.

**Table 5** Percent shoot and root organogenesis of *Stevia rebaudiana* in agar and other low-cost substitutes to agar gelled media.

| Agar Substitutes | Amount Used (g/L) | Percent Shoot Induction | Percent Root Induction |
|---|---|---|---|
| Agar | 20 | $66.6 \pm 2.7^{b}$ | $100.0 \pm 3.78^{a}$ |
| Sago | 110 | $64.6 \pm 4.1^{b}$ | $100.0 \pm 4.11^{a}$ |
| Laundry starch | 90 | $23.3 \pm 1.3^{e}$ | $9.93 \pm 0.17^{e}$ |
| Corn starch | 90 | $42.2 \pm 2.2^{cde}$ | $100.0 \pm 4.76^{a}$ |
| Isubgol | 28 | $100.0 \pm 3.2^{a}$ | $40.0 \pm 3.32^{d}$ |
| Agar + semolina | 10+45 | $39.4 \pm 1.1^{de}$ | $60.0 \pm 3.22^{c}$ |
| Agar + sago | 10+55 | $30.5 \pm 2.5^{de}$ | $100.0 \pm 5.21^{a}$ |
| Agar + laundry starch | 10+45 | $26.1 \pm 1.08^{e}$ | $75.0 \pm 2.69^{b}$ |
| Agar + corn starch | 10+45 | $50.0 \pm 3.3^{bcd}$ | $100.0 \pm 2.88^{a}$ |
| Agar + Isubgol | 10+14 | $62.5 \pm 4.07^{bc}$ | $80.0 \pm 4.15^{b}$ |
| LSD ($P \leq 0.05$) | | 22.39 | 13.33 |

*3.5. Benefit cost ratio and other properties of the agar and agar substitutes*

Different botanical starches were used as alternative to commercial grade agar for stevia propagation in laboratory. These substitutes reduced the agar cost by 90% (sago) followed by 88.75% reduction in cost while using corn starch and 83.2% when isubgol was used as gelling agent. Semolina was the cheapest agar substitute reducing cost up to 97.7% but no response was induced in the explant when used alone. Like semolina laundry starch was also inferior in its performance and reduced the cost to 88.7%. Wide use of agar is because of its property of gel clarity, proper gelling ability and non-toxicity during cultures, its stability over periods of long cultures and its resistance to metabolism. But higher price of agar leads us to find some cheaper alternatives to it. All the agar substitutes provided proper gelling up to some extent, but in comparison to agar, media were hazy (poor gel clarity), it was difficult to identify contamination, root emergence was difficult to detect. Some of the gelling agents (cornstarch, laundry starch) started a foul smell after few weeks of culture. Isubgol gelled properly but culturing operations, media preparation and sub culturing were a little difficult to handle because of its mucilaginous property. Media prepared with sago was also hazy, detection of contamination during root growth was a problem but sustained cultures for a longer time period (Table 6).

**Table 6** Comparative cost of different gelling agents with respect to their use in a media of equal proportions.

| Gelling Agents | Quantity g/L | Price USD/kg | Price USD/L | Cost Reduction Over Control (%) |
|---|---|---|---|---|
| Agar (control) | 20.0 | 34.56 | 0.69 | 0 |
| Semolina | 90.0 | 0.17 | 0.016 | 97.75 |
| Sago | 110.0 | 0.62 | 0.068 | 90.1 |
| Laundry Starch | 90.0 | 0.86 | 0.078 | 88.75 |
| Corn Starch | 90.0 | 0.86 | 0.078 | 88.75 |
| Isubgol | 28.0 | 4.15 | 0.12 | 83.2 |
| Agar + Semolina | 10+45 | 17.37 | 0.35 | 48.875 |
| Agar + Sago | 10+55 | 17.59 | 0.38 | 45.05 |
| Agar + Laundry starch | 10+45 | 17.71 | 0.38 | 44.375 |
| Agar + Corn Starch | 10+45 | 17.71 | 0.38 | 44.375 |
| Agar + Isubgol | 10+14 | 0.40 | 0.40 | 41.6 |

## 4. Discussion

Composition of culture media is very important for the success of growth and development of plants, cell tissue and organ cultures in *in-vitro* condition. As a gelling agent, agar is one of the most important components of the media that is added to support explant by increasing the viscosity of the medium [13]. Agar has been used since very long time for tissue culture as well as for microbial cultures. Reasons, which tempted us to find alternatives to agar were its wide use over time, limited resources, and its higher cost depending upon the quality of the agar [11-13, 15]. Low cost tissue culture technology is much needed approach for large scale propagation of plantlets. For that purpose, low cost substitutes to laboratory grade agar have a great potential. We tested certain low cost easily available alternatives of agar and got significant differences in results. Norhayati et al. [16] also found that combination of cheaper agar substitutes with agar has promising results as compared with agar used alone in the medium. A study on banana varieties by Saraswathi et al. [17] indicated that early sprouting of shoots occurred in isubgol gelled medium as compared with their control medium. Strength of the gel, its clarity, stability on high temperature etc. are the main factors that represent the quality of the agar or any gelling agent [15]. It is apparent from the current results that components of a media and its viscosity greatly influenced the shoot and root organogenesis. Similar results were concluded by Ullah et al. [18] using different substitutes of agar for orchid propagation and isubgol gelled media was found the most effective one. However, Arthur et al. [13] conducted a study by using different solidifying agents and response of isubgol was found poorer as compared to other solidifying agents. On the other hand, Sharifil et al. [19] reported maximum organogenesis during micropropagation of African violet in media gelled with agar alone. Findings of Saraswathi et al. [17] contradicts

these findings, which are actually in accordance with our current results. The findings of Shailaja and Patil, [20] are consistent with the current results indicating sago as the best substitute to agar. Rishsi [21], observed better root organogenesis on medium solidified with isubgol as compared to agar gelled medium. Bhattarcharya and Bhattarcharya [22], also confirmed superiority of isubgol through their findings. Recently Prabhuling et al. [23] also obtained similar results indicating better rate of propagation (quality wise) on medium solidified with sago in comparison to the one solidified with commercial grade agar. Better results of isubgol and sago might be due to physical consistency and polysaccharide nature of these starches.

Best response of isubgol gelled media could be due to its higher mucilage content (>30%). On the other hand, sago is composed of starch, sugar, minerals, fiber and calcium. Upon heating, starch present in sago converts into dextrin (complex polysaccharide). Presence of these polysaccharides and nutrients in medium provide better environment for growth and proliferation, which makes sago a better substitute to agar in media [19-24]. Naik and Sarkar [25] also reported equal performance of potato micro plants on sago and agar gelled media. But the poor results of some agar substitutes might be due to poor gelling ability as well as presence of inhibitory substances present in them. This fact has also been confirmed by Arthur et al. [13] that some gelling agents have inhibitory effect on morphogenesis because of the presence of inhibitory substances. Agar is extracted through different mechanical processes from species of red sea weeds. It is widely used as gelling agent because of its stability during long periods of culture, best gelling property, high gel clarity and resistivity to metabolism. But some scientist indicated that agar contains certain compounds (inhibitors) and their presence have inhibitory effect on the development and differentiation of tissues being cultured on medium solidified with agar [26]. Better performance of sago and isubgol confirms the findings of Arthur et al. [13], who suggested the presence of additives (peptones, salts) in agar substitutes, which plays an important role in enhancing the growth of Micropropagated plantlets. Presence of these polysaccharides and nutrients in medium provides better environment for growth and proliferation [25]. Sago even having poor gel clarity and weak gelling ability showed the longest shoots and roots emergence in comparison with agar. This could be because of higher rate of nutrient uptake influenced by the rate of water influx into the tissue in the *in vitro* condition [24, 25]. In that case, agar gelled medium has negative potential of water (lower) than its other substitutes [26].

Lower uptake of water because of the matrix potential results in lower nutrient uptake, ultimately resulting in poor growth of micro propagated plantlets. Although antagonized the current findings, who obtained the highest regeneration 123/frequency on agar and starch combined growth medium. In the present study, corn starch was the quickest to induced rooting response. Jain and Babar [15] also confirmed the current results by obtaining early root initiation on mediums solidified with botanical starches than agar. Starches provide large amount of carbohydrates (35%) and some mineral matter (1%), which act as an additional source of carbon to the growth medium [27], that could be the possible reason for early induction in starches. Agar comes in variety of packages and vary from brand to brand in its purity and gelling properties, poor results obtained on agar alone and in combination with other substitutes, might be due to presence of impurities and inhibitors present in it. Ozel et al. [28], George et al. [29] and Ivanova and Staden [30], reported the adverse effects of agar like growth inhibition, cytotoxic effect, necrosis and hyperhydricity.

Takahito et al. [31] also confirmed the findings by obtaining a greater number of roots on medium solidified with agar substitutes than on agar. Possible reason for obtaining different response in terms of root numbers on different gelling agents could be because of differences in their osmotic effects along with their varying nutritional composition. Higher ionic concentration of sago has also been reported by Kuria et al. [32]. Furthermore, it was documented that cell division is also influenced by medium, which contain high number of ionic materials. But they observed increase in root numbers in media gelled with isubgol, which is partially in contrast with our current results. Shailaja and Patil [20, 33] documented poor growth of roots in isubgol gelled medium and considered poor nutrient absorption as well as the hard gelling of media as possible reasons. Kuria et al. [32], concluded that isubgol performs poorly in case of rooting at all concentrations, which could be because of poor nutrient diffusion, which limits their availability and subsequently reduce growth. Failure of stevia to induce roots in media solidified with semolina could be because of higher carbohydrates concentration. Perata et al. [34] and Rahman and Blake [35], documented that high concentration of sugar affects organogenesis and growth of tissues by negatively affecting the signal transduction pathway, suppressing the effect of growth hormones. Buah [36] reported satisfactory root induction percentage and afterward performance of plantlets in media solidified with combination of agar with starch. Findings of are synergistic with our current findings who obtained better performance of low-cost alternatives of agar, in every aspect as compared to laboratory grade agar [37]. Expansion of starches in tissue culture media is of great importance because it enhances availability of water, PGRs and nutrients to the plantlets [37]. However, the plantlets regeneration and the productivity of secondary metabolites is not restricted to any specific component of the culture media. Many biotic and abiotic elicitors and other components also fluctuate the regeneration potential and the synthesis of metabolites of interest. The supplementation of silver nanoparticles (400 uM) enhanced the growth responses and the biosynthesis of rebaudioside-A content in higher quantities than control in the regenerants of *Stevia rebaudiana* (Bert.) [38]. In another study, the synthetic seeds obtain from alginate encapsulation of shoot tips, improve germination, proliferation and elongation of shoots; root initiation, elongation and multiplication on culture media without PGRs and produce true-to-type plantlets of *Stevia*

*rebaudiana* (Bert.) after 30 days [39]. The addition of meta-Topolin to culture media enhanced the multiplication potential of *Stevia rebaudiana* (Bert.) with maximum shoots initiation and proliferation rate as compared to other cytokinins, and also enhanced the biosynthesis of rebaudioside-A content than mother plant [40].

## 5. Conclusion

*S. rebaudiana* (Bert.) is one of the emerging sweet plant with multiple applications in various industries. The self-incompatibility, infertile and heterozygous seeds, lower pollination potential interrupt the normal prorogation of stevia plants. Such plants need biotechnological method to enhanced the large-scale propagation to overcome the market demand. Plant tissue culture is one of the best strategies to produce true-to-type and uniform plantlets in very short time. For tissue culture, MS media are widely use to obtain maximum results. However, plant tissue culture is expensive as compared to conventional propagation. It is better to use alternatives in MS media to reduce the cost of plant tissue cultured-derived plants. One of the most expensive component of MS media is agar. In this study, multiple agar alternatives were exploited to compared the cost with agar-grown plantlets. It is concluded from the current results that a significant variation was observed in shoot and root organogenesis exposed to various gelling agents. Among these gelling agents Isubgol, sago and corn starch were found effective in shoot and root organogenesis and its related studied attributes. The addition of agar substitutes reduced media cost in comparison with up to 90% by sago, 88.75% by corn starch and 83.2% by Isubgol. These results suggest that these agar alternatives should be used instead of agar. Semolina was the least expensive and reduced media cost by 97.75% but it was failed to induce shoot and root organogenesis. Like semolina laundry starch was also inferior in its performance and reduced the cost to 88.7%. It is concluded from the current experiment that agar alternative plays a key role in cost reduction during the micropropagation of *S. rebaudiana* (Bert.). It is concluded from the results that mass propagation of stevia and other medicinal or herbaceous plants can be achieved by using agar alternatives which reduce the cost of production of high-quality plant material characterized by genetic uniformity and free of contamination and can be scale up to bioreactor to produce metabolites of interest.

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