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Proliferation of embryogenic callus of Satoimo taro (*Colocasia esculenta* var. *antiquorum*) in culture media with various levels of sucrose and gelling agent

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Abstract. *Fitriani H, Aryaningrum PD, Hartati NS. 2016. Proliferation of embryogenic callus of Satoimo taro (*Colocasia esculenta var. *antiquorum) in culture media with various levels of sucrose and gelling agent. Nusantara Bioscience 8*: *316-320.* Satoimo taro (*Colocasia esculenta* var. *antiquorum*) is one of the promising varieties of taro which have potentially high value to cultivated. Tissue culture or in vitro culture considers an economically useful technique to mass propagate this variety of taro. The research was conducted to find out the optimum media for proliferation success of embryogenic callus of Satoimo. The explants used embryogenic callus of 12-months-old storage materials were obtained from the previous study. Proliferation media include a basal half-strength MS salt (Murashige and Skoog) enriched with of 0.1 mg/L TDZ, 0.05 mg/L 2.4 D, and 100 mg/L L-glutamine. Adding to the basal media, we set the treatments by combining two factors i.e. Sucrose with three level of concentration i.e. 3, 4, and 5% which cross-tabulated with three gelling agents, i.e., Agarose, Phytagel, and Gerlite. The research parameters included the percentage of callus diameter growth, the color and callus discoloration, and the structure of callus. The data recorded periodically every 7 days in one month. Data were analyzed using analysis of variance and followed by Duncan Multiple Range Test (DMRT) to determined the significant difference ($p<0.05$) among the treatments. The result revealed that the callus diameter growth has the greatest percentage at medium with a combination of Micro agar and sucrose 3%. The Micro-agar enhanced the presence of callus with yellow color. Phytagel combined with a high percentage of sucrose (5%) indicated a browning properties of callus. Gelrite cause the callus with discoloration appearance (white).

Keywords: Embryogenic callus, in vitro culture, proliferation, Satoimo taro

INTRODUCTION

Satoimo taro, known as root crop, is herbaceous plant of Araceae family which commonly refers to the plant *Colocasia esculenta*. Other species of taro include Alocasia, Cyrtosperma, and Xanthosoma. Taro is cultivated all over the tropics and subtropics regions and planted in height around 0 - 1400 asl. It becomes a staple root crop for all over communities worldwide, particularly in majority of Asia, Pasific Island, West Africa, and Amazonian regions of south America. Indonesia is one of the important centre of taro domestication. The demand for Satoimo increases, which can be seen from the high increase of Satoimo utilization in food industry and also the high demand of this roots overseas especially in Japan.

In vitro somatic embryogenesis is an important prerequisite for the use of many biotechnological tools for genetic improvement. The technique could have a great impact on high economically important plant and horticulture such as the guava, *A. malaccensis Lam*. (Akhtar 2013; Saikia et al. 2012; Lambardi et al. 2013). In modern breeding, somatic embryogenesis is an important system for vegetative propagation. It can be used to generate new plants from single cells.

Recently, somatic embryos has attracted attention in plant biotechnology, because it provides useful systems to produce transgenic plants, as well as material for the production of artificial seeds (Jafarzadeh-Bajestani et al. 2011; Aswathi 2012). The technique has become an effective tool for genetic transformation purposes. Furthermore, embryogenic tissues have been proved as the best cell source for transgenic plant regeneration and also germplasm preservation through somatic cryopresevation in plant breeding program (Martinelli 1997). In this regard, embryonic structure becomes a preferred material for plant genetic improvement, because it has been known as a single cell, thus; somatic embryo derived-plant is easier to be controlled. Moreover, the regeneration of somatic embryogenesis may reduce the formation of chimeras (Suprasana et al. 2012).

Based on the totipotency theory, the capability of the single plant cell to regenerate become intact plant can widely open opportunities to mass propagation, rapid proliferation, high quality of regeneration, the shorten waiting of the following reproductive season, resistance to diseases, nursery saving and germplasm conservation (Devi et al. 2014). Bajaj (1995) stated that somatic embryos are ideal materials for both short-term and long-term storage of plant germplasm due to their bipolar structure, which enable to be germinated as new plant seedling anytime.

Several factors are affecting the success of somatic embryogenesis. These factors, including the genotype of

plant explant, medium formulation, plant growth regulators, and physiological characteristic of cells. According to Lizawati (2012), the successful outcome of plant micropropagation via somatic embryogenesis can be seen from the high percentage of embryogenic callus formation in the explant cultured in particular medium. The nutrient composition and substances added to the medium is considered as the primary factor in effecting the successful work of tissue culture (Basri 2008). Furthermore, another factors that also important in making in vitro medium are gelling agents and sucrose. The gelling agent most used in in vitro studies is agarose, a polysaccharide generally extracted from seaweed. According to Saad and Elshahed (2012), agarose contains a low percentage Ca, Mg, K, and Na.

As most parts of the plants or explants cultured into a glass medium (in vitro) caused the high evaporation level which lead to the low rate of photosynthesis. Therefore, tissue culture or in vitro plants need enough carbohydrates as an energy source. Sugar or sucrose is used as an alternative energy source applied in the culture medium. Sucrose is the best energy-producing carbohydrate than other sources like lactose, maltose, galactose, mannitol and technical sugar (Saad and Elshahed 2012; Placide et al. 2012). Besides a source of energy, sugar also serves as the osmotic pressure of the media. According to Rahman et al. (2009), carbohydrates also had affect on the physiology, growth, and plant cell differentiation.

In order to develop the potential used of taro as food and industry materials, then the objective of the study was to determine the proliferation level of embryogenic callus of Satoimo taro cultured in media with various concentration of sucrose and gelling agents. The best formulation obtained from this research can be used for embryogenic callus proliferation of Satoimo taro, which can further provide the seeds in large quantities to support national food security and overseas demand.

MATERIALS AND METHODS

The research was conducted between January 13, and February 7, 2014 at Laboratory of Plant Molecular Genetics and Biosynthetic Pathway Alteration, Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia.

Explant source and medium

The experiment used explant from 12-months-old (12 mo) storage collection of embryogenic callus of Satoimo taro. The selection of explant in this age category to find out the capacity of the callus to proliferated after 1 year storage period (). The basal medium used a half-strength MS salt (Murashige and Skoog) supplemented with 0.1 mg/L TDZ, 0.05 mg/L 2,4-D, and 100 mg/L L-glutamine. Explants were cultured in sterilized aseptic treatment mediums i.e. a basal medium with the addition of various types of gelling agent (Micro agar, Phytagel, and Gerlite) in combination with several level of sucrose (3, 4, dan 5%). The sterilization procedure used autoclave in 121^{o}C (1.5 atm) for 20 minutes.

The responses of callus regarding the percentage of diameter growth, the color and discoloration, and the structure of callus were observed periodically every 7 days in one month. The data were analysis by analysis of variance (ANOVA) and followed by Duncan multiple range test to determined the significant difference ($p<0.05$) among the means.

RESULTS AND DISCUSSION

The diameter of callus

Callus growth was influenced not only by genotype and hormones but also by gelling agents and sucrose concentration. Agar concentrations for tissue culture media ranged from < 3 to 15 g·L^{-1} (Debergh 1983; George and Sherrington 1984; Miller and Murashige 1976; Singha 1982), depending on the type of agar. Based on statistical analysis, the average diameter of callus in cassava was not significantly different after it was cultured in media containing various sucrose concentration and agar (Table 1). Based on the Table 1, results showed that the low concentration of carbon sources was more effective on callus maintenance for Micro agar and Phytagel. In the last observation (4 weeks), Micro agar with the concentration of 3% increased more callus diameter of cassava than those in the concentration of 4% and 5%. This result is in agreement with See et al. (2011), who reported that the increased level of sucrose around 60g/Lmedia or higher, would decline the cell growth of plants. Similar trend was reported by Sato et al. (1996) who mentioned that the supplementation of sucrose concentration more than 0.09 M into cell suspension media decreased the cell growth of strawberry. It may attribute to the inhibition of nutrient uptake due to the increased levels of the osmotic potential or the high viscosity of the cell suspension medium. Another type of agar, Phytagel with the high concentration levels could reduce the diameter of cassava. Zhang et al. (2010), however, stated that Phytagel used as the gelling agent in the somatic culture medium of upland catton could improve the quality of the callus and embryogenic callus. Otherwise, the average of diameter callus is increased when it cultured on medium containing Gelrite and the high concentration levels of sucrose. In another study, Jaramillo and Summers (1990) found that the media containing 3% of Gelrite was the best media formulation for producing the optimum results of tomato anther culture.

Table 2 showed that gelling agents influence the growth of calluses especially in callus diameter despite not significant. According to Babbar and Jain (1998), Agar is the most commonly used gelling agent in plant tissue culture, followed by Gellan gum or Gelrite®, a polymer of glucuronic acid, hamnose, glucose and O-acetyl moieties (Scholten and Pierik 1998). Agar has a function as water binding agent, so that the higher concentration of agar in the culture media will cause the stronger binding water capacity.This is different to another type of gelling agents,

Gelrite, which require the presence of cations for gelation process. Other advantages of using solidifying agents are its stability in all feasible incubation temperatures, its undigested ability to plant enzymes, and its persistence to media constituents (George and Sherrington 1984).

Sucrose has been identified as the best carbon source to support callus maintenance followed by mannitol, fructose and glucose. Highest proliferation of callus was also observed in media containing sucrose. This might happen because sucrose is easily proceed in metabolism (Abu et al., 2005). Sucrose is usually added to a culture medium as energy source so that tissues do not depend on photosynthetic activity for their primary growth (Islam and Ichihashi 1999). In addition, sucrose has a correlation with the osmotic potential of the medium (Nowak et al., 2004) which result in the high absorption of mineral nutrients contained in medium. Interestingly, those mineral nutrients are essential to the cells growth. Of the reasons, the optimal osmotic pressure is required for optimal proliferation purposes. Ganapathi et al. (1999) also reported that banana callus cultured into medium supplemented with 3% of sucrose could rapidly proliferate to semi-friable embryogenic callus (90-95%), compared to those cultured in mannitol which result in nodular and compact formation of callus (60-65%).

The effects of sucrose concentration in MS medium on callus production could be measured from the changes of callus diameter. Callus cultured in the medium containing 5% sucrose would produce the largest size of callus. On the contrary, callus cultured in 3 and 4% sucrose concentration did not have effect on the average diameter of callus. Overall, based on the Table 3, effects of different concentrations of sucrose was non significantly different with the average callus diameter in all various agar.

The color of callus

The response of explants in a culture medium can be detected by the color of callus. This feature is useful in describing physiological performances of the callus cells i.e. survival feature of callus. The callus formation usually possessed the various visual color (Figure 1). According to Peterson and Smith (1991), the embryogenic callus is characterized by morphological appearance i.e a yellowish-white color, bright or clear appearance, and friable form. On the contrary, the non-embryogenic callus has a pale color, brownish-yellow, soft and watery, and compact formation (not easily to be separated in to aggregates).

The color of embryogenic callus was clearly observed under a stereomicroscope. Based on Figure 2, the color of all callus was varied in both the pretreated callus and other callus cultured in treatment media i.e. white, yellow and brown. The color of callus grown in Micro-agar media appeared on transparent-white callus formation in all concentration levels of sucrose. However, callus had a initial browning formation when it was cultured in medium containing of 5% sucrose. Interestingly, it subsequently turns to yellow color in percentages less than 80%. However, after cultured in treatment media with the same gelling agent (Micro-agar) and supplemented with 5% sucrose, most callus maintain their yellow performances with the percentage reached to 100%. In addition, there is no browning indication in the callus. In media with Micro-agar, the percentage of callus with yellow color formation reached to 100% when it was cultured in the media supplemented with 4% sucrose. It was indicated that most of the sucrose addition in culture media might be utilized by callus for the growth purpose, so that it has a less possibility to bind with the Micro-agar. Thus, the callus can sufficiently supplied nutrition from media.

As mentioned by George and Sherrington (1984), sucrose is a useful organic-based carbon source used to cell development. The presence of sucrose in sufficient dosage can support many cell activity, including cell division, enlargement, and differentiation. Similarly, Husin et al. (2004) stated that an appropriate dosage concentration of sucrosa supplemented in culture media would enhance the division and enlargement of the cells that collectively increase callus growth due to all cells use energy from the sucrose.

The different result was obtained in the treatment with Phytagel as geling agent. There is no indication of browning pigmentation of callus in all treatments. But after it was cultured in the media with the addition of 5% sucrose the browning pigmentation was formed. The browning phenomenon in the callus grown in medium with high sucrose concentration might happen due to the increase level of osmotic pressure in the media with high sucrose dosage. High osmotic pressure caused cell division and nutrient uptake of the explant slowly proceed, which lead to the callus growth retardation (Marlin at al. 2012).

Tabel 1. Means of callus diameter of cassava (cm) cultured in the media containing different type of gelling agents and the concentration of sucrose

Gelling agents	Concentration of sucrose		
	3%	4%	5%
Micro agar	1.43 cm	1.21 cm	1.38 cm
Phytagel	1.33 cm	1.39 cm	1.19 cm
Gelrite	0.78 cm	0.86 cm	1.34 cm

Tabel 2. Mean callus diameter of cassava (cm) in various gelling agents

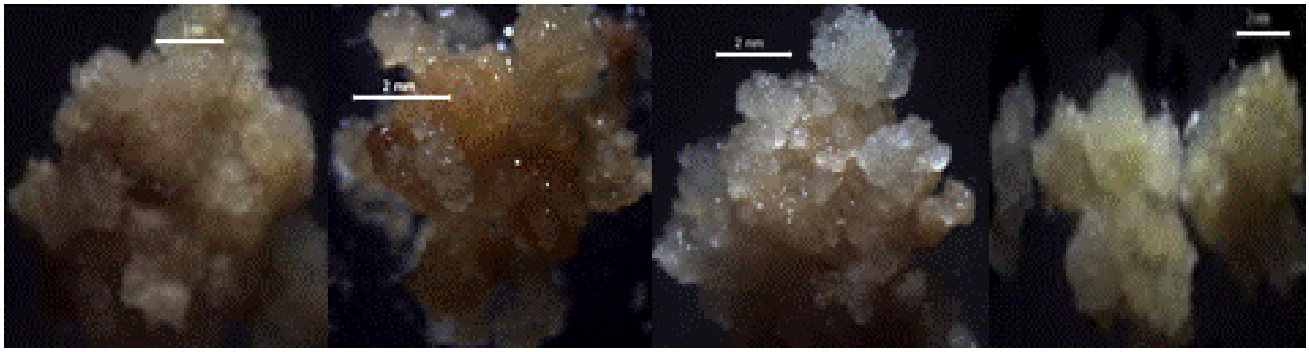
Gelling agents	(cm) (ns : not significant)
Micro agar	1.340 ± 0.059
Phytagel	1.303 ± 0.666
Gelrite	1.340 ± 0.059

Note : ns: not significant P > 0.05

Tabel 3. Mean callus diameter of cassava (cm) in various concentration of sucrose

Concentration of sucrose	Mean callus diameter of cassava (cm) (ns : not significant)
3%	1.180 ± 0.202
4%	1.153 ± 0.156
5%	1.303 ± 0.057

Note : not significant P > 0.05



A B C D

Figure 1. The color of embryogenic callus of 12-months-old storage Satoimo Taro before a treatment. There were four different color i.e. A. Light-brown, B. Dark-brown, C. White, D. Yellow. Bar = 2 mm

This is in agreement with Lizawati (2012) who reported that the color of callus turning to dark-brown might indicate the reduction of callus growth performances. Similarly, Santoso and Nursandi (2001) said that the browning pigmentation of callus correlated to the high sucrose and carbohydrate content in the medium. The browning formation was apparently caused by a synthesis of secondary metabolites, such as phenolic substances. Wattimena (1991) pointed out that the phenolic substances have constrained effects on cell division, cell expansion and the growth of callus. Furthermore, a prolong-culture of callus in this media would induce explant deaths. As reported by Yildiz et al. (2007) who conducted research in sugar beet found that the phenolic substances tend to increase in pararel with the high sucrose content of a medium. In contrast result obtained in treatment with lower concentration of sucrose i.e. 3% and 4% gave of yellow color in callus performance. In the last observation, the 3% sucrose gave 80% of yellow color in callus performance and the 20% of the remaining callus was in transparent-white formation. In another treatment, the 4% sucrose still covered 60% of callus in yellow performances and the remaining was in transparent-white and light brown performances with its percentages reaching to 20% and 10%, respectively.

In the media solidified with Gelrite revealed that 10%, of the callus formation was in brown color when it was cultured in media with the addition of sucrose content 4% and 5%. But, when it was cultured in media containing of 3% sucrose, the color of callus was 100% in yellow color formation. However, in last observation, most callus turned to transparent-white in color and a less part of them becoming light-brown in color when it was cultured in media supplemented with various levels of sucrose. According to Zhang et al. (2010), Gelrite can be used to reduce a browning formation in callus. Because Gelrite has an ability to increase a relative humidity (RH), inducing the vitrification in the callus. Gelrite usage also did not cause a browning pigmentation in the callus despite the high concentration of sucrose was added. In media with the addition of Gelrite and 3% sucrose (G3), the percentage of callus appeared in white and brown color performances was less than 20%, while the yellow one was about 80%. In media with the addition of Gelrite and sucrose 4% (G4), the yellow color of callus decreased to 70%, while the white callus color increased to 18% without browning indication. In media supplemented with Gelrite and sucrose 5% (G5), callus did not undergo browning formation but it tended to form a light-brown callus which increased up to 18%, and the remaining callus was in constant color.

The structure of callus

Table 4 showed the structure of 12-mo Satoimo taro embryogenic callus observed under stero-microscope. All callus formed a friable structure. The structure of callus could be categorized into two formation i.e. the compact and friable callus. The good quality of callus had been generally characterized by friable structure. The friable callus could be clearly observed from the intercellular connection, which apparently has a loose connection; thus, they easily separated into many single cell aggregates. Conversely, the compact callus performances possessed the tight connection in the intercellular structure. Pierik (1987) states that the structure of the callus can vary from compact to friable, depending on the type of plant material, the composition of the nutrient in the media, the plant growth regulators and environmental culture conditions.

In conclusion, the 12-mo embryogenic callus explant of Satoimo taro could be positively proliferated which it could be seen from the increase size of callus diameter. Micro-agar used in culture medium could increase the callus formation, which is mostly in yellow color without browning formation and less occurrence of white color callus. Phytagel with 5% sucrose could induce browning formation in callus. Meanwhile, Gelrite affected on the white-color callus and reduced the yellow-color callus in all sucrose dosage, as well as prevented callus from browning pigmentation.

Table 4. The structure of 12-mo embryogenic callus of Satoimo taros grown in various treatment media

Gelling agents	Sukrosa concentration		
	3%	4%	5%
Micro agar	Friable	Friable	Friable
Phytagel	Friable	Friable	Friable
Gelrite	Friable	Friable	Friable

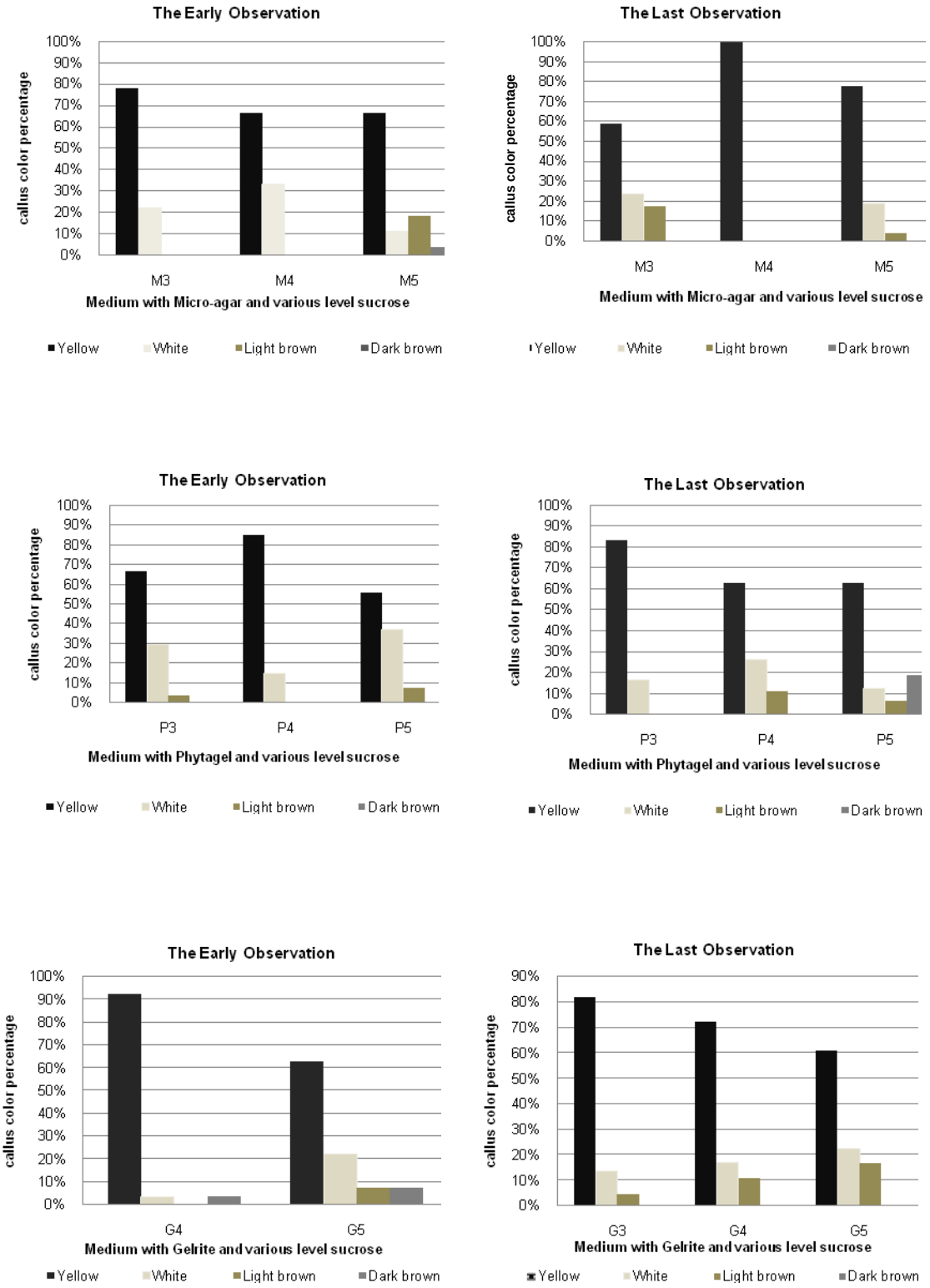


Figure 2. The changed color in 12- months-old embryogenic callus of Satoimo Taros in various gelling agents and different level of sucrose. M3 (Micro-agar with 3% sucrose), M4 (Micro-agar with 4% sucrose), M5 (Micro-agar with 5% sucrose), P3 (Phytagel with 3% sucrose), P4 (Phytagel with 4% sucrose), P5 (Phytagel with 5% sucrose), G3 (Gelrite with 3% sucrose), G4 (Gelrite with 4% sucrose), G5 (Gelrite with 5% sucrose).

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