

## Mass propagation of *Vaccinium corymbosum* in bioreactors

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### Summary

Two kinds of bioreactors were evaluated for *in vitro* propagation of *Vaccinium corymbosum*: a temporary immersion system (RITA®) and a permanent immersion reactor. Results were compared with the system currently used of micropropagation in semi-solid medium, used as control for this study. Two varieties, 'O'Neal' and 'Georgia Gem', of the triple hybrid *Vaccinium corymbosum*, *V. ashei* and *V. darrowii* were employed. Explants were cultured in WPM medium (Lloyd and Mc Cown, 1981) containing 0.01mM ascorbic acid, 0.22mM adenine sulphate, 2% sucrose and 20µM 2iP. To minimize hyperhydricity of explants, the effect of ancymidol (0.4, 0.9 and 2.0 µM) as growth retardant and phloroglucinol (0.32mM) as promoter of lignin biosynthesis was tested. The highest multiplication rates were 28 and 24 shoots per bud for the varieties 'O'Neal' and 'Georgia Gem' respectively, and were achieved in the permanent immersion system containing phloroglucinol. Light microscopy of the shoots showed a more developed and lignified xylem in the phloroglucinol-derived plants. Shoots obtained from the multiplication system that were 3 or more centimeters long were rooted *ex vitro* in a mixture of soil, peat and perlite (1:1:1) resulting in 80% rooting. Plants acclimatized well and exhibited normal development in the greenhouse.

**Abbreviations:** IBA – Indol-3-butyric acid, 2iP – 6-( $\gamma$ ,  $\gamma$ -dymethylamino) purine, WPM – Woody Plant Medium (Lloyd and McCown, 1981).

**Key words:** ancymidol, blueberry, hyperhydricity, micropropagation, phloroglucinol

### Resumen

## Propagación masal de *Vaccinium corymbosum* en bioreactores

Se evaluaron dos tipos de bioreactores para la propagación *in vitro* de *Vaccinium corymbosum*: un reactor de inmersión temporal (RITA®) y un reactor de inmersión permanente. Los resultados fueron comparados con el sistema de micropropagación habitualmente empleado de multiplicación en medio de cultivo semi-sólido, usado como control en este trabajo. Se emplearon dos variedades, 'O'Neal' y 'Georgia Gem', del triple híbrido *Vaccinium corymbosum*, *V. ashei* y *V. darrowii*. Los explantos fueron cultivados en medio WPM (Lloyd y Mc Cown, 1981) suplementado con ácido ascórbico (0,01 mM), sulfato de adenina (0,22 mM), sacarosa (2%) y 2iP (20µM). Para minimizar la ocurrencia de hiperhidricidad en los explantos se evaluó el efecto del ancymidol (0.4, 0.9 y 2.0 µM) como retardante del crecimiento y del phloroglucinol (0.32mM) como promotor de la biosíntesis de lignina. Las máximas tasas de multiplicación obtenidas fueron 28 y 24 brotes por yema para las variedades 'O'Neal' y 'Georgia Gem' respectivamente, las cuales fueron alcanzadas en el sistema de inmersión permanente con phloroglucinol. Observaciones al microscopio óptico de las plantas obtenidas mostraron una mayor diferenciación de xilema y mayor lignificación de los tejidos en los explantos provenientes del tratamiento con phloroglucinol. Los brotes provenientes del tratamiento de multiplicación que alcanzaron 3 cm o más de altura fueron enraizados *ex vitro* en una mezcla de tierra, turba y perlita (1:1:1) obteniéndose un 80% de enraizamiento. Las plantas fueron aclimatadas y mostraron un desarrollo normal en invernáculo.

**Abreviaciones:** IBA – ácido indol 3 butírico, 2iP – 6-( $\gamma$ ,  $\gamma$ -dymethylamino) purine, WPM – Woody Plant Medium (Lloyd y McCown, 1981).

**Palabras clave:** ancymidol, blueberry, hiperhidricidad, micropropagación, phloroglucinol

## Introduction

An increasing interest exists in Uruguay for alternative fruit species, among which the great demand for blueberry (*Vaccinium corymbosum*) for the establishment of commercial orchards stands out. *V. corymbosum* is an important fruit crop in many northern latitude countries. Its high anthocyanin content is an excellent source of antioxidants, which are believed to reduce the risk of various human degenerative diseases (Prior, 2000). According to United States Food and Drug Administration, blueberries are low in fat, very rich in antioxidants, vitamins C, K and A as well as iron and manganese, and a good source of dietary fiber. They are consumed both as fresh and processed products.

Research carried out by INIA (Instituto Nacional de Investigación Agropecuaria) concludes that Uruguay has adequate soils and climate for the cultivation of this species, with the additional advantage that we can supply fruit to satisfy the high demand from Europe and United States out of season, from October to December.

The main limitation for establishing its culture is the lack of a propagation system which produces disease-free plant material for commercial plantations. Traditionally this species is clonally propagated by rooting cuttings, but this method cannot assure enough identical disease-free plant material (González, 2000). Being a relatively new species for cultivation in Uruguay, micropropagation is the best alternative to rapidly satisfy the current demand of plants.

Micropropagation allows the fast clonal propagation of plants free from pathogens, and is routinely used to produce large numbers of fruit, forestry, and ornamental species. Blueberries have been micropropagated for more than twenty years from nodal segments of adult field-grown plants (Cohen and Elliot, 1979; Zimmerman and Broome, 1980). However, the extent to which micropropagation can be practiced commercially is limited by the cost of production, due to the intense manipulations required in the different stages of the process (George, 1993).

Automation of micropropagation in bioreactors has been advanced by several authors as a possible way of reducing costs of micropropagation. Bioreactors provide a sterile environment, designed to strongly promote

development and allowing a maximum control of microenvironment conditions (Paek and Hahn, 2002). Bioreactors provide a rapid and efficient plant propagation system for many agricultural and forestry species, utilizing liquid media to avoid intensive manual handling. Bioreactor cultures have several advantages compared with agar-based cultures, with a better contact of the plant tissue with the culture medium, and optimal nutrient and growth regulator supply, as well as aeration and medium circulation, the filtration of the medium and the scaling-up of the cultures (Ziv, 2005). The main disadvantage imposed by the use of liquid medium in bioreactors is the development of hyperhydric plants, which present abnormal morphogenesis such as vitreous leaves, stomata malfunction and hypolignification among others (Ziv, 1991).

The objective of this study was to optimize a bioreactor multiplication system for blueberry to satisfy the actual demand of high quality plant material. Two types of bioreactors were evaluated: a permanent reactor and a temporary immersion system RITA® (Teisson, 1996).

## Materials and methods

### Plant material

Two varieties, 'O'Neal' and 'Georgia Gem', of the triple hybrid *Vaccinium corymbosum*, *V. ashei* and *V. darrowii* were employed in this study. Both varieties show low to medium cold requirements for development, which is an appropriate condition for the temperate climate of Uruguay.

The source of explants was axillary shoots, 3-5 cm long, with 3-4 buds each, grown on agar-solidified multiplication medium.

### Multiplication medium

The multiplication medium employed was WPM (Lloyd and McCown, 1981) containing 0.01 mM ascorbic acid, 0.22 mM adenine sulphate, 2 % (w/v) sucrose and 20  $\mu$ M 2iP, at pH 5.5. The same medium composition but solidified with 0.7 % (w/v) Difco Bacto-agar was used as control treatment in all experiments.

To avoid the occurrence of vitrification two strategies were employed: addition of Ancymidol to the medium in three concentrations: 0.4; 0.9 and 2.0  $\mu\text{M}$ , to evaluate its effect as growth retardant, inhibiting gibberellins biosynthesis (Thakur, 2006); and to prevent the occurrence of vitrification, the effect of adding phloroglucinol (0.32 mM) to the medium as lignin precursor was also evaluated (George, 1993).

### Multiplication system

Two kinds of bioreactors were evaluated: a permanent reactor and a temporary immersion system (RITA® Teisson, 1996).

The permanent immersion reactor was built with a 2.0 liter Nalgene® vessel with screw lid and two ports to allow filtered air inlet and outlet. Aeration was supplied by a 31 liter Somar compressor at an average of 550  $\text{ml}\cdot\text{min}^{-1}$  for each bioreactor. Two volumes of media (500 and 1000 ml) were evaluated, and 15 explants were placed in each bioreactor.

RITAs were aerated with an IKAWI pump AP-115, ten explants each, and three volumes of media (200, 250 and 300 ml). The system was programmed for immersions of 1 min. every four hours.

Cultures were incubated at 20° C for two months, with a photon flux of 20  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 16 h photoperiod.

Multiplication treatments are shown in Table 1.

### Histological analysis

Histological differences of solid and liquid media-derived shoots were evaluated by light microscopy in shoots (4 mm diameter or more) stained with Toluidine blue.

### Rooting and transfer to soil

Microshoots (3 cm or longer) were rooted *ex vitro* in plastic trays with a mixture of soil, peat and perlite (1:1:1 in volume). The trays supply a microenvironment with relative humidity nearly 100%. The effect of a rapid immersion in IBA (500 and 1000 ppm) was evaluated.

### Statistical analysis

All experiments were performed three times. Each experiment consisted of at least three replicates (reactors).

Multiplication rate (number of micro shoots obtained per bud) and bud size (mm) were measured weekly for two months.

**Table 1.** Multiplication treatments.

Treatment	Culture system	Volume of media (L)	Ancymidol ( $\text{mg}\cdot\text{L}^{-1}$ )	Phloroglucinol ( $\text{mg}\cdot\text{L}^{-1}$ )	Aeration
BR1	Bioreactor	1	-	-	24h
BR2	Bioreactor	1	0.1	-	24h
BR3	Bioreactor	1	0.25	-	24h
BR4	Bioreactor	1	0.5	-	24h
BR5	Bioreactor	1	-	40	24h
BR6	Bioreactor	0.50	-	-	24h
BR7	Bioreactor	0.50	-	40	24h
BR8	Bioreactor	1	-	-	NO
BR9	Bioreactor	0.50	-	-	NO
R1	RITA®	0.20	-	-	1min/4h
R2	RITA®	0.25	-	-	1min/4h
R3	RITA®	0.30	-	-	1min/4h

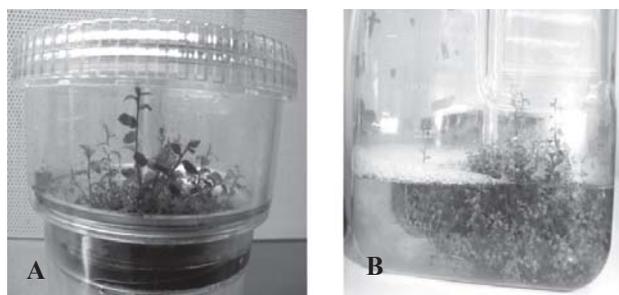
BR: permanent immersion bioreactor, RITA®: temporary immersion reactor. Agar solidified media (7% w/v) was used as control in all experiments.

Results of the experiments were analyzed with STATISTICA® using analysis of variance and least significant difference (LSD) means separation.

## Results and discussion

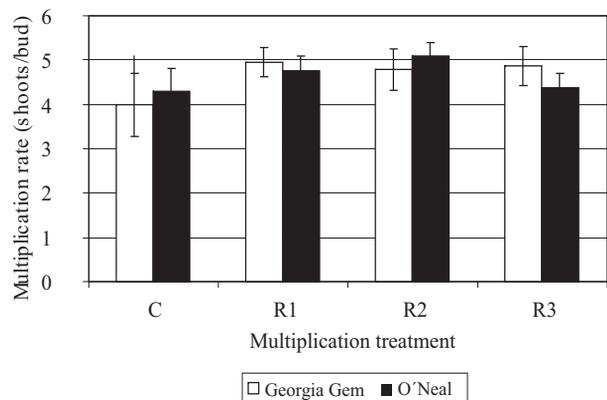
### Shoots proliferation

Shoot proliferation was achieved with both multiplication systems (Figure 1). Explants obtained with both culture systems were green, vigorous and apt for rooting. Multiplication rate in all treatments was higher or equal than in the control using semi-solid medium.



**Figure 1.** Blueberry shoots proliferation in two multiplication systems. (A) RITA®, (B) Permanent immersion bioreactor.

Figure 2 shows the multiplication rates obtained with the three volumes of media employed in the RITA® system (200, 250 and 300 ml) and the control. The results presented show that there was no significant

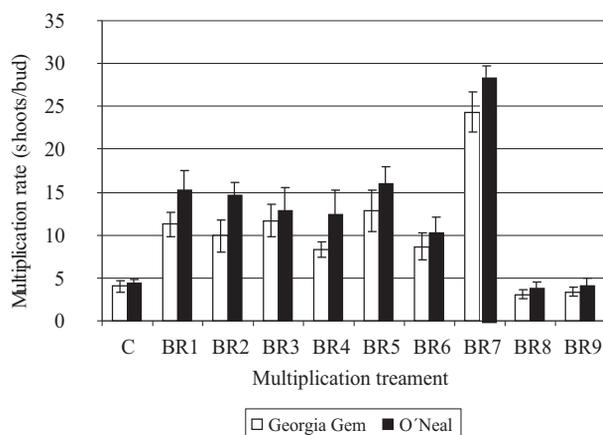


**Figure 2.** Blueberry multiplication rate in the RITA® system. C: control in semi solid medium; R1, R2 and R3: RITA® with 0,20; 0,25 and 0,30 litre of media. Different letters indicate significant differences at  $p = 0,05$  by the LSD Test.

effect of media volume (R1, R2 and R3 respectively) on multiplication rate. Moreover, the volumes tested did not differ from the control. This could be due to the frequency and duration of the immersion period used. Krueger *et al.* (1991) demonstrated the importance of immersion frequencies for the proliferation of serviceberry shoots. In this system, the period of immersion is determinant of the capacity of absorption the explants will have, and therefore a key factor in promoting multiplication. Too short or separated periods of immersion may not be enough to achieve the desired effect of enhancing multiplication rate and development of new axillary buds. On the other hand, too frequent immersion periods may result in vitrified shoots. In addition, the optimum immersion time varies depending on genotype (Etienne and Berthouly, 2002). The shoots obtained in our experiment did not show symptoms of vitrification, but the low rates of multiplication achieved with all the volumes tried, justify the need for further evaluation of frequencies and duration of immersion periods.

Permanent immersion bioreactors (BR 1-9) were more adequate for the multiplication of the blueberry varieties used in this study with multiplication rates from 8 to 24 and 10 to 28 microshoots per bud for 'Georgia Gem' and 'O'Neal' respectively (Figure 3).

Multiplication rate in the permanent immersion bioreactor was significantly superior to the RITA® system, which didn't show differences with the semi-solid system of multiplication.



**Figure 3.** Blueberry multiplication rate in the permanent immersion bioreactors. C: control in semi solid medium; BR1 – BR9: bioreactor treatments. Different letters indicate significant differences at  $p = 0,05$  by the LSD Test.

‘O’Neal’ showed a tendency to higher multiplication rates than ‘Georgia Gem’ in the permanent bioreactors, although differences were not always significant. This behavior is similar to the one observed on semi-solid medium for this variety (data not shown).

Results presented here show a strong effect of the type of reactor employed on the multiplication rate of both materials. Only in the case of bioreactors without aeration (BR 8 and 9) multiplication rate did not differ significantly from the control. For the bioreactors with aeration (BR 1-7) the lowest multiplication rate doubled that of the control.

### Bud length

Table 2 shows average final length of buds after two months treatment. Buds in the RITA system (R1, R2 and R3) showed better development, achieving sizes of 6 mm for the variety ‘Georgia Gem’. However, this was inversely related with the multiplication rate, which was similar to semi-solid medium for the RITA system. Similar results were reported with *Allium sativa* by Kim *et al.* (2004). These authors obtained a higher rate of multiplication and better quality micro-shoots with the permanent immersion system.

### Effect of phloroglucinol to prevent hyperhydricity

The addition of phloroglucinol to the culture media, besides preventing the occurrence of hyperhydric shoots, determined the highest rates of multiplication when working with smaller volumes.

Figure 4 shows hyperhydric blueberry explants obtained without aeration (BR8). The effect of adding phloroglucinol to the culture medium was markedly significant (less plants developed hyperhydric shoots) when working with a volume of 0.5 litres. Figure 5 shows a cluster of microshoots obtained with treatment 7, from one initial explant with 4 buds, which provided a multiplication rate of 24 shoots per bud for ‘Georgia Gem’.

Phloroglucinol is used to prevent hyperhydricity in micropropagation for its effect in lignin biosynthesis providing precursors which normally are poorly or not synthesized at all in hyperhydric tissues. Certain enzymes, particularly p-coumarate:CoA ligase, a key enzyme in lignin synthesis, show significantly less activity in vitrified explants. Adding phloridzin or its precursor phloroglucinol to the culture medium of apple and sunflower shoots prevented the occurrence of

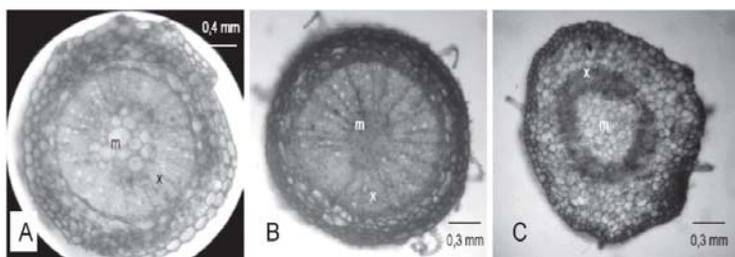
**Table 2.** Final bud length.

Treatment	Culture system	Aeration	Avg. bud length (mm)	
			‘Georgia Gem’	‘O’Neal’
CONTROL	Semi-solid	-	3.00 ± 0.25a	2.50 ± 0.32a
BR1	Bioreactor	24h	3.03 ± 0.29a	2.10 ± 0.22a
BR2	Bioreactor	24h	3.03 ± 0.21a	2.20 ± 0.27a
BR3	Bioreactor	24h	3.26 ± 0.64a	2.03 ± 0.28a
BR4	Bioreactor	24h	1.45 ± 0.26b	1.55 ± 0.21b
BR5	Bioreactor	24h	3.06 ± 1.77a	2.30 ± 0.53a
BR6	Bioreactor	24h	2.11 ± 0.25a	2.20 ± 0.41a
BR7	Bioreactor	24h	2.59 ± 0.57a	3.78 ± 0.51c
BR8	Bioreactor	NO	0.90 ± 0.02c	1.21 ± 0.28b
BR9	Bioreactor	NO	1.65 ± 0.07d	1.82 ± 0.09a
R1	RITA®	1min/4h	4.48 ± 0.84e	4.52 ± 0.21d
R2	RITA®	1min/4h	4.00 ± 0.89e	3.97 ± 0.36cd
R3	RITA®	1min/4h	6.05 ± 3.02e	4.15 ± 0.34cd

Data are expressed as mean + standard error.  
Mean separation in column by LSD,  $p < 0,05$ .



**Figure 4.** Hyperhydric blueberry explants, bioreactor without aeration (BR8).



**Figure 6.** Cross section of shoots, variety O'Neal. (A) Semi-solid medium, (B) Liquid medium with phloroglucinol, BR7, (C) Liquid medium without phloroglucinol, BR1. m: medulla; X: xylem.



**Figure 5.** 'Georgia Gem' microshoots cluster after two months with 40mg.l<sup>-1</sup> Phloroglucinol (BR7).

hyperhydricity, increasing the activity of enzymes involved in lignin synthesis (George, 1993).

Working with a volume of 1 liter, treatments were not significantly different, though the same tendency was observed. This could be explained by the effect that volume has on aeration, which could be limiting when working with large volumes. Depending on the species, optimal volume media: air relation may vary, and is determinant of oxygen availability for the tissues. In all experiments, although hypoxia is never reached and the explants multiply successfully in 1 liter (reaching multiplication rates of 11 and 15, 'Georgia Gem' and 'O'Neal' respectively), smaller volumes clearly result more favorable for this species, allowing a better contact of the explants with the air and therefore a better diffusion of oxygen. In order to confirm the effect of phloroglucinol as lignin precursor, shoots from treatment 7 and the control were studied by light microscopy and

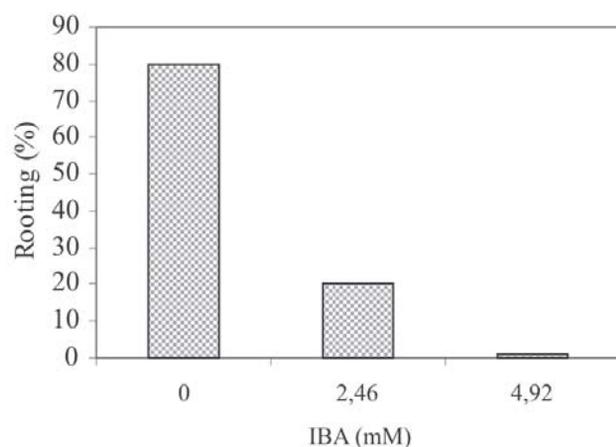
compared with semi-solid medium derived shoots. Shoots from treatment 7 (0.32mM phloroglucinol) had more lignified tissues than the control (shown by staining), with a better development of xylem, similar to the shoots from semi-solid medium (Figure 6).

### Effect of Ancymidol

Ancymidol is reported in the literature as a growth retardant, and has been used to reduce vitrification and promote shoot clusters (Maki, 2005; Thakur, 2006). This compound inhibits the kaurene oxidation sequence of reactions in the gibberellins biosynthesis pathway. However, in the concentrations tested in our experiments, Ancymidol did not improve or even inhibited multiplication rate (Figure 3, BR 2, 3 and 4). Similar results were cited by other authors in Narcissus (Chen and Ziv, 2004), where Ancymidol had a clear effect in promoting vitrification, against what was expected. These results could be explained by the concentrations evaluated, which could be too low to promote cluster formation for this species, considering that endogenous hormones levels differ greatly amongst species.

### Rooting and Acclimatization

Micropropagated shoots formed roots within four weeks in a 1 peat: 1 perlite: 1 soil mixture. The highest rooting percentage (80%) was obtained in the control treatment without IBA. Plants were vigorous and showed an intense green colour. Both IBA concentrations tested



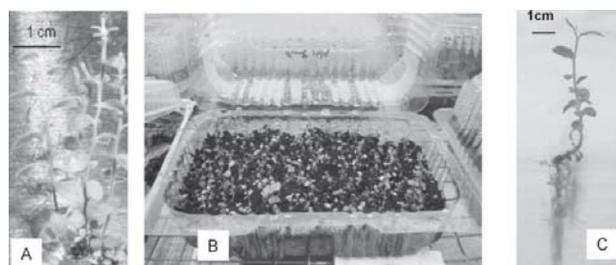
**Figure 7.** Effect of IBA concentration on the percentage of rooting. Different letters indicate significant differences at  $p=0.05$ .

resulted in very poor (2.46 mM IBA) and no rooting (4.92 mM IBA), as shown in Figure 7. Moreover, the highest auxin concentration used had a negative effect on the microshoots, which became brown and necrotic. This could be partly explained by the effect of phloroglucinol, which may have a synergic effect with endogenous levels of auxins, therefore the exogenous addition of IBA resulting inhibitory of root differentiation. *Ex vitro* rooting conditions for 'Georgia Gem' are shown in figure 8. Plants acclimatized well and exhibited normal development in the greenhouse. These results show that bioreactor-derived microshoots are apt for *ex vitro* rooting, thus avoiding the need for *in vitro* rooting which is more work-consuming.

## Conclusions

This is the first report on the use of permanent immersion bioreactors for the micropropagation of *Vaccinium corymbosum*.

The results indicate that for clonal propagation *in vitro* of the blueberry varieties included in this study, the permanent immersion system results in multiplication rates not yet obtained with semi-solid protocols. Plant quality (size, hardiness, survival *ex vitro*), for the two varieties evaluated was similar or better than for plants grown in semi-solid media. The high multiplication rates achieved resulted in a biomass increase of six-fold in eight weeks, without need for periodic transfer of explants to fresh medium. This represents an effective reduction of costs, where intensive manual handling is the main component. An



**Figure 8.** *Ex vitro* rooting experiment, variety 'Georgia Gem'. (A) Microshoots apt for rooting, (B) Microshoots in plastic trays without IBA, (C) Rooted microshoot after 30 days in greenhouse.

additional reduction in costs derives from the use of liquid medium, avoiding the use of agar, one of the major components of medium cost.

Addition of phloroglucinol as precursor in the lignin biosynthesis pathway resulted in effective control of hyperhydricity and best multiplication rate, associated with better quality and high survival percentage of microplants. This system thus has great potential for *in vitro* production of blueberry plants commercially, to satisfy the current demand of plant material at lower costs.

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