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Response of root explants to *in vitro* cultivation of marketable garlic cultivars

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ABSTRACT

Garlic cultivars are sexually sterile under standard growth conditions, with direct implications for commercial production costs as well as breeding programs. Garlic is propagated commercially via bulblets, which facilitates disease transmission and virus load accumulation over vegetative generations. Tissue culture produces virus-free clones that are more productive, while keeping the desired traits of the cultivar. Consequently, this technique allows studies of garlic genetics as well as guarantees genetic conservation of varieties. We aimed at analyzing the *in vitro* regeneration of eight marketable cultivars of garlic using root segments as explants. For each genotype, bulblet-derived explants were isolated and introduced into MS medium supplemented with 2,4-D and 2-iP. Calli were transferred to MS medium supplemented with 8.8 μM BAP and 0.1 μM NAA (regeneration medium A), or with 4.6 μM kinetin alone (regeneration medium B). The calli were then evaluated for regeneration frequency after sixty days of *in vitro* cultivation. The noble cultivar 'Jonas' presented the highest rates of plant regeneration among the cultivars tested. The medium A, which contained auxin and cytokinin, induced the highest regeneration rates of all cultivars. The process described herein is simple, reproducible and can potentially be used as a tool in molecular breeding strategies for other marketable cultivars and genotypes of garlic.

Keywords: *Allium sativum*, bulb, callus induction, organogenesis, regeneration, tissue culture.

RESUMO

Resposta dos explantes radiculares ao cultivo *in vitro* de cultivares comerciais de alho

Cultivares de alho são sexualmente estéreis sob condições padrão de cultivo, com implicações diretas nos custos de produção comercial, bem como em programas de melhoramento. O alho é comercialmente propagado por meio de bulbilhos, o que facilita a transmissão de doenças e leva ao acúmulo de cargas virais ao longo das gerações. A cultura de tecidos produz clones livres de vírus que são mais produtivos, mantendo as características desejadas da cultivar. Consequentemente, esta técnica permite estudar a genética do alho, bem como garantir a conservação genética das variedades. Nosso objetivo foi analisar a regeneração *in vitro* de oito cultivares comerciais de alho usando segmentos de raiz como explante. Para cada genótipo, explantes derivados de bulbilho foram isolados e introduzidos em meio MS suplementado com 2,4-D e 2-iP. Calos foram transferidos para meio MS suplementado com 8,8 μM BAP e 0,1 μM NAA (meio de regeneração A), ou com 4,6 μM cinetina somente (meio de regeneração B). Os calos foram avaliados quanto à frequência de regeneração após sessenta dias de cultivo *in vitro*. A cultivar nobre 'Jonas' apresentou as maiores taxas de regeneração entre as cultivares testadas. O meio de cultura A, o qual continha auxina e citocinina, induziu as maiores taxas de regeneração em todas as cultivares. O processo aqui descrito é simples, reprodutível e pode ser usado potencialmente como uma ferramenta em estratégias de melhoramento para outras cultivares comerciais e genótipos de alho.

Palavras-chave: *Allium sativum*, bulbo, indução de calos, organogênese, regeneração, cultura de tecidos.

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Garlic (*Allium sativum*) is a monocot species of the Alliaceae, cultivated mainly in temperate zones around the globe. The *Allium* genus contains more than 500 members, and garlic is thought to have originated in Central Asia (Etoh *et al*., 2001). Brazil is one of the largest garlic markets in the world, with most of its garlic production commercialized *in natura*; yet, there is a trend to increase industrial garlic processing. In 2010, garlic production reached 17.7 million metric tons worldwide (www.faostat.org), with China as the leading producer (13.7 million metric tons), encompassing almost 80% of world production. In South America, Argentina is the largest producer, at 128,900 tons. Brazil produced 104,586 tons of garlic in 2010, ranking at the 13th position. Among the thirty-five leading producers in the world, Tajikistan ranks first in yield (300 t ha^{-1}), China is in fourth place (205 t ha^{-1}), and Brazil ranks at number 20 (99.2 t ha^{-1}). The world's average yield in 2010 was 132 t ha^{-1}, which indicates that many countries can largely improve yield and production to fulfill the genetic potential of garlic production. However, yield increase will require serious attention to crop management, while making use of optimal agricultural practices and conducting breeding programs to optimize genetic fitness for local cultivation conditions.

Traditionally, garlic breeding programs have been limited to clonal selection of mutant genotypes, since the almost totality of the species germplasm is sexually sterile. Tissue culture and plant transformation techniques via particle bombardment and *Agrobacterium tumefaciens* of garlic have been developed, thereby allowing the use of these advances for propagation and breeding programs. Garlic sterility has implications not only for breeding programs, but also directly affects production costs, since garlic cultivation requires expensive vegetative propagules ('cloves') for propagation and enables disease transmission. An efficient method for mass propagation of garlic is therefore highly desirable (Ayabe & Sumi, 1998). In this regard, the best vegetative propagation method for garlic is using plants originating from *in vitro* culture, which produces bulbs that are free of viruses, and other diseases and pests.

Somatic embryogenesis and organogenesis have long been studied in garlic (Abo El-Nil, 1977; Nagasawa & Finer, 1988; Ayabe *et al*., 1995; Ayabe & Sumi, 1998; Myers & Simon, 1998, 1999; Barandiaran *et al*., 1999a, b; Kondo *et al*., 2000; Robledo-Paz *et al*., 2000; Sata *et al*., 2000; Luciani *et al*., 2001; Fereol *et al*., 2002; Luciani *et al*., 2006; Xu *et al*., 2008; Lee *et al*., 2009; Yanmaz *et al*., 2010). Histological studies on several Alliaceae members (leeks, onions and garlic) have revealed different callus types associated with plant regeneration (Eady *et al*., 1998; Fereol *et al*., 2002; Zheng *et al*., 2003; Luciani *et al*., 2006; Lee *et al*., 2009). The most important factors affecting plant regeneration are explant type, the physiological condition of the explant, genotype and the growth regulator combination used in the culture medium. Suh & Park (1995) used abnormal roots derived from garlic anthers, pedicels, and bulblets as explants to regenerate plantlets. Root tips are also commonly used for garlic regeneration, with or without a callus phase (Barandiaran *et al*., 1999a, b; Robledo-Paz *et al*., 2000). Haque *et al*. (1997, 1999) obtained up to 75% frequency of direct shoot regeneration from root tips. Regarding callus differentiation and plant development determination by growth regulators, several reports have shown the effects of the synthetic auxins, picloram (4-amino-3,5,6-trichloropicolinic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), on different garlic cultivars (Barandiaran *et al*., 1999b; Myers & Simon, 1999; Robledo-Paz *et al*., 2000; Sata *et al*., 2000; Fereol *et al*., 2002; Zheng *et al*., 2003; Luciani *et al*., 2006).

Genetic improvement programs and genetic research will largely benefit from efficient protocols for garlic plant transformation. So far, only four reports have been published on garlic genetic transformation via the *Agrobacterium* system. To produce transgenic plants, Kondo *et al*. (2000) used a vector bearing the report (*uid*A) and hygromycin (*hpt*) selection genes on callus explants. Zheng *et al*. (2004) introduced insect-resistance genes (*cry1Ca* and *H04* derived from *Bacillus thuringiensis*) onto five garlic lines using calli originated from root explants. Eady *et al*. (2005) describe an *Agrobacterium* genetic transformation protocol, which uses immature embryos of garlic and leek (*Allium ampeloprasum* var. *porrum*). More recently, Kenel *et al*. (2010) have proposed a modified protocol from the one originally described by Eady *et al*. (2000), which uses immature leaf tissues via direct regeneration of somatic tissues. This protocol reduces the cultivation period and the possibility of somatic mutations. Herein, we aimed at establishing an efficient *in vitro* regeneration protocol for marketable varieties of garlic using root segments as explants. We also aimed at exploring the variability of *in vitro* responses among different varieties of garlic.

MATERIAL AND METHODS

Plant material- Eight cultivars of garlic (*A. sativum*) from various Brazilian market groups (noble, semi-noble, common) were classified according to their vegetative cycle length, commercial appearance and mean number of bulblets per bulb. The cultivars used were Amarante-Embrapa, Roxinho 5063, IAC75 – Gigante de Curitibanos, IAC63 – Mexicano Br and Lavínia 1632 (semi-noble group, medium cycle); Cajuru 2315 (common, early cycle); Cateto Roxo (common, medium cycle); and Jonas (noble group, which requires vernalization). Plant material was obtained from the Germplasm collection at the Instituto Agronômico de Campinas, Campinas, São Paulo state, Brazil.

Culture media - The basic culture medium consisted of full strength MS salts, vitamins (Murashige & Skoog, 1962) and 30 g L^{-1} sucrose, with the pH adjusted to 5.8 with KOH prior to the addition of 2 g L^{-1} Phytagel®, and then autoclaved.

***In vitro* root induction -** Bulblets were peeled from their protective leaves and immersed in 70% ethanol for five minutes, followed by disinfection in 2.5% sodium hypochlorite (with two drops of Tween 20 per 100 mL solution) for twenty minutes under constant stirring and ten successive rinses in autoclaved distilled water. Each explant was then excised to expose its 1 cm apical meristem region and each explant was introduced to a single glass tube (25 x 125 mm) with a 20 mL basic MS medium (introduction medium). The explants were placed in a growth chamber at 27±2ºC with a 16 h light period supplied by fluorescent white lamps at ~31 μmol m^{-2} s^{-1} for four weeks.

Callus induction - Roots originating in the base of the bulblet after four weeks of *in vitro* cultivation were then used for callus induction. One-centimeter root segments were cut and transferred to the callus induction medium (a basic MS medium supplemented with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D); 0.5 μM N^6-2-isopentenyl adenine (2-iP); and 0.2 g L^{-1} casein hydrolysate), as described by Zheng *et al.* (2003). Each Petri dish with a 25 mL medium contained twenty-five root segments and each cultivar was represented with ten plates. Cultures were kept in the dark at 27±2ºC for two months, when callogenesis was recorded. Subcultures were transferred to fresh media every four weeks.

Plant regeneration - For comparison, calli with 8 mm^2 originating from root segments were transferred to

containers with two distinct regeneration media: medium A, according to Kondo *et al*. (2000), and medium B, according to Zheng *et al*. (2003). Both media consisted of a basic MS medium (vitamins and salts). Medium A consisted of MS supplemented with 8.8 µM 6-benzylaminopurine (BAP) and 0.1 µM alpha-naphthaleneacetic acid (NAA), while medium B comprised MS supplemented with 4.6 µM N^6-furfuryladenine (kinetin). Explants were incubated at 27±2°C during a 16-hour photoperiod and transferred to a fresh medium every two weeks. Incubation times varied according to the regeneration medium used: Kondo *et al*. (2000) reported five months of incubation, whereas Zheng *et al*. (2003) only incubated explants for two months. The calli were evaluated for shoot regeneration rates, and the two regeneration protocols were compared.

Statistical analysis - For shoot regeneration efficiency, a 2 x 8 factorial design was used (two media and eight garlic cultivars) in a completely randomized experimental design with five replicates. Each replicate was represented by one Petri dish containing twenty-five explants. Data were evaluated using SAS software (SAS Institute Inc., USA) for ANOVA and, when statistically significant, Tukey's test was applied at 5% probability.

RESULTS AND DISCUSSION

Callus induction - Root segments were tested for callogenesis, and shown to be responsive explants. Callogenesis was characterized by an initial elongation of the explant, tissue swelling and formation of small callus punctuations in the edges along the whole explant, followed by massive division of undifferentiated cells. The cultivars Cateto Roxo, Cajuru 2315 and Roxinho 5063 did not produce any callus on the callus induction medium at day thirty, but were responsive upon transfer to fresh media, at which point (eight weeks in culture) these explants started to dedifferentiate and form calli. Callogenesis in the other cultivars (Amarante-Embrapa, IAC75 – Gigante de Curitibanos, IAC 63 – Mexicano Br, Lavínia 1632 and Jonas) was visible at day thirty, and upon transfer to fresh media, they presented vigorous growth. All cultivars showed callogenesis, although at different rates and with distinctive appearances. For 'Jonas', out of 2,500 root segments introduced, more than 1,300 calli were produced, ranking first among all cultivars. 'IAC75 – Gigante de Curitibanos' ranked second with more than 600 calli (Figure 1). According to Zheng *et al*. (2003), the sole use of auxin in the callus induction medium results in a high callogenesis frequency when compared to the auxin/cytokinin combination. This high induction, however, later resulted in a low regeneration rate. Zheng *et al*. (2003) also reported callus induction rates of up to 56% and shoot regeneration of only 6.6% when treated with auxin alone, in comparison with 33% callus induction and 31% shoot regeneration when auxin was combined with cytokinin. In this regard, De Klerk *et al*. (1997) and Guohua

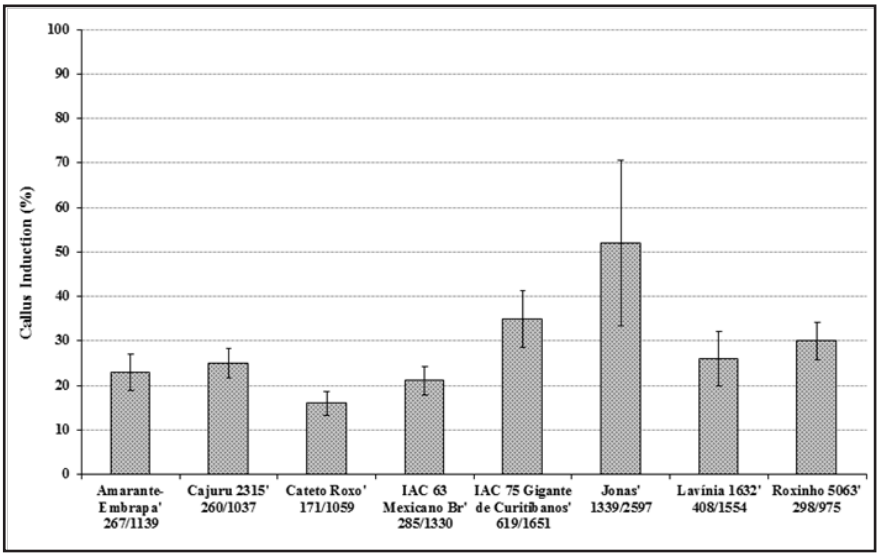

Figure 1. Rate of callogenesis induction in root segments of eight marketable garlic cultivars after 60 days. The percentages were calculated by the number of explants producing calli over the total number of explants introduced in each cultivar. Ten independent replicates were carried out and the average percentages were calculated. The number of introduced explants varied among cultivars due to the availability of plant material, root formation responsiveness and experiment contaminations. The fractions below each variety's name correspond to the total number of responsive calli (numerator) and the total number of calli studied (denominator). Piracicaba, CENA/USP, 2011.

Table 1. Effect of the two regeneration media on callus regeneration in eight garlic cultivars. Piracicaba, CENA/USP, 2011.

Cultivars	Responsive calli (n°)[1]	
	Medium A	Medium B
Jonas	3.57 a	2.43 a
Amarante-Embrapa	2.57 b	1.50 ab
IAC 63-Mexicano Br	2.43 b	1.57 ab
Cajuru 2315	2.43 b	1.86 ab
IAC 75-Gigante de Curitibanos	2.29 b	1.57 ab
Cateto Roxo	2.14 b	2.14 ab
Roxinho 5063	2.14 b	1.29 b
Lavínia 1632	2.00 b	1.57 ab

[1]Averages followed by different letters indicate statistically significant differences according to Tukey's test (p>0.05). Medium A (Kondo *et al*., 2000); Medium B (Zheng *et al*., 2003).

Table 2. Indirect *in vitro* organogenesis regeneration of eight marketable garlic cultivars. Piracicaba, CENA/USP, 2011.

Regeneration media	Responsive calli (nº)[1]
MS + 8.8 µM BAP + 0.1 µM NAA[2]	2.45 a
MS + 4.6 µM kinetin[3]	1.74 b

[1]Averages followed by different letters indicate statistically significant differences according to Tukey's test (p>0.05); [2]Medium A (Kondo *et al*., 2000); [3]Medium B (Zheng *et al*., 2003).

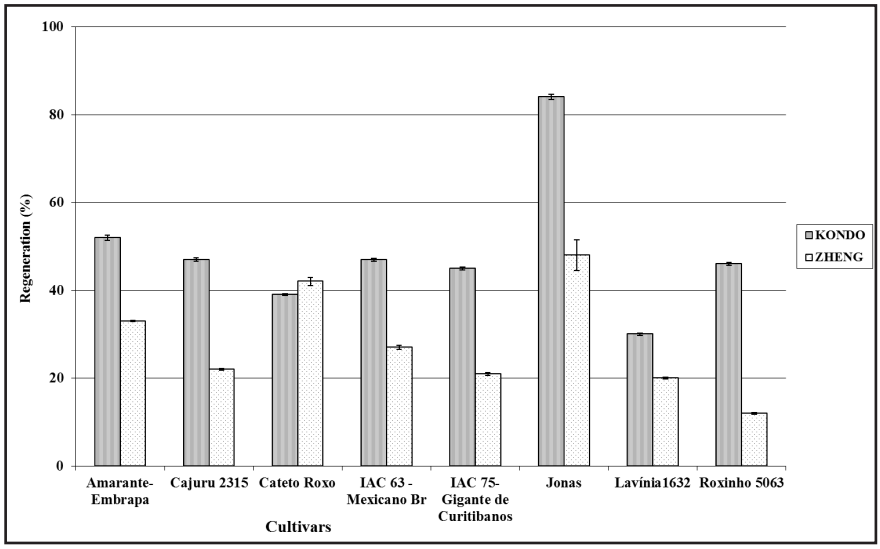

Figure 2. Callus regeneration (%) in eight garlic cultivars after five months on culture medium A (Kondo *et al*., 2000) and after two months on culture medium B (Zheng *et al*., 2003). Piracicaba, CENA/USP, 2011.

(1998) reported that auxins induce callus formation and proliferation as well as somatic embryogenesis, while cytokinins induce mostly shoot and root differentiation and elongation. In keeping with these findings, the auxin/cytokinin combination in the callus induction medium proposed by Zheng *et al*. (2003) resulted in an acceptable number of calli for all cultivars analyzed in this study. Our study therefore confirms that this protocol is genotype-independent.

Plant regeneration - According to Robledo-Paz *et al*. (2000), the use of root segments as explants highly increases the potential for garlic regeneration. According to Barandiaran *et al*. (1999a), genotype influences *in vitro* responsiveness, suggesting that protocols should be optimized for each cultivar. In garlic, *in vitro* organogenesis occurs indirectly when explants are cultivated in the dark, which provides conditions for callus formation and regeneration of adventitious meristems from callus cells. In our conditions, calli were formed after sixty days of cultivation on callus induction media containing either a combination of auxin and cytokinin, or cytokinin alone. Analyses of variance revealed statistical differences for culture media as well as for cultivars, but found no significant interaction between these two factors. Statistical analyses revealed differences in callus regeneration between the media used. The medium supplemented with cytokinin and auxin promoted the best rates of regeneration for all cultivars, as proposed by Kondo *et al*. (2000). Overall, 'Jonas' showed the highest rate of callus regeneration (84%) (Figures 2 and 3). Callus regeneration rates for the genotypes 'Jonas', 'Amarante-Embrapa' and 'IAC63 – Mexicano Br,' were more vigorous than for the other genotypes, particularly given the development of shoots and leaves (Table 1). Based on these results, we compared the cultivar responses for each medium, with medium A presenting an average of 2.45 responsive calli per plate, while medium B showed an average of 1.74 (Table 1 and 2). These values might be considered low in terms of *in vitro* regeneration; however, medium A showed regeneration rates 40% higher than medium B. In the regeneration medium supplemented with cytokinin alone (medium B), 'Jonas' also presented the highest regeneration rate (48%), although lower than its rate for medium A. For medium B, the rate of 'Jonas' differed significantly from that of 'Roxinho 5063', which ranked second for this parameter.

Zheng *et al*. (2003) noticed that a small amount of cytokinin (0.5 µM 2-iP) in the callus induction medium had a stimulating effect on regeneration. The regeneration frequency for media containing auxin alone varied between 6.6% and 8.9%, whereas the combination of auxin plus cytokinin resulted in 30% to 48% regeneration. Interestingly, the cultivar 'Jonas' consistently showed better regeneration than any other cultivar tested, i.e., independently of supplementation by growth regulators in the callus induction medium (Table 1).

In summary, the noble cultivar, 'Jonas' presented the highest rate of plant regeneration among the cultivars tested, and the regeneration medium supplemented with auxin and cytokinin (Kondo *et al*., 2000) led to the highest regeneration rates for all cultivars. The process herein described is simple, reproducible and can potentially be used for other marketable cultivars and genotypes of garlic for purposes of clonal mass propagation and viral clean up. It can also serve as the basis for producing transgenic garlic for genetic studies, or to be incorporated as a tool in molecular breeding strategies.

The germplasm used in the present study represented various types of Brazilian cultivars. Although these cultivars performed differently upon *in vitro* culture conditions, the regeneration system reported here is efficient, reliable, and cultivar-independent. Based on previous research using onion, shallot and garlic calli as explants for transformation (Zheng *et al*., 2001, 2003, 2004), root segment calli from both apical and non-apical tissues are an appropriate starting point for garlic genetic transformation via

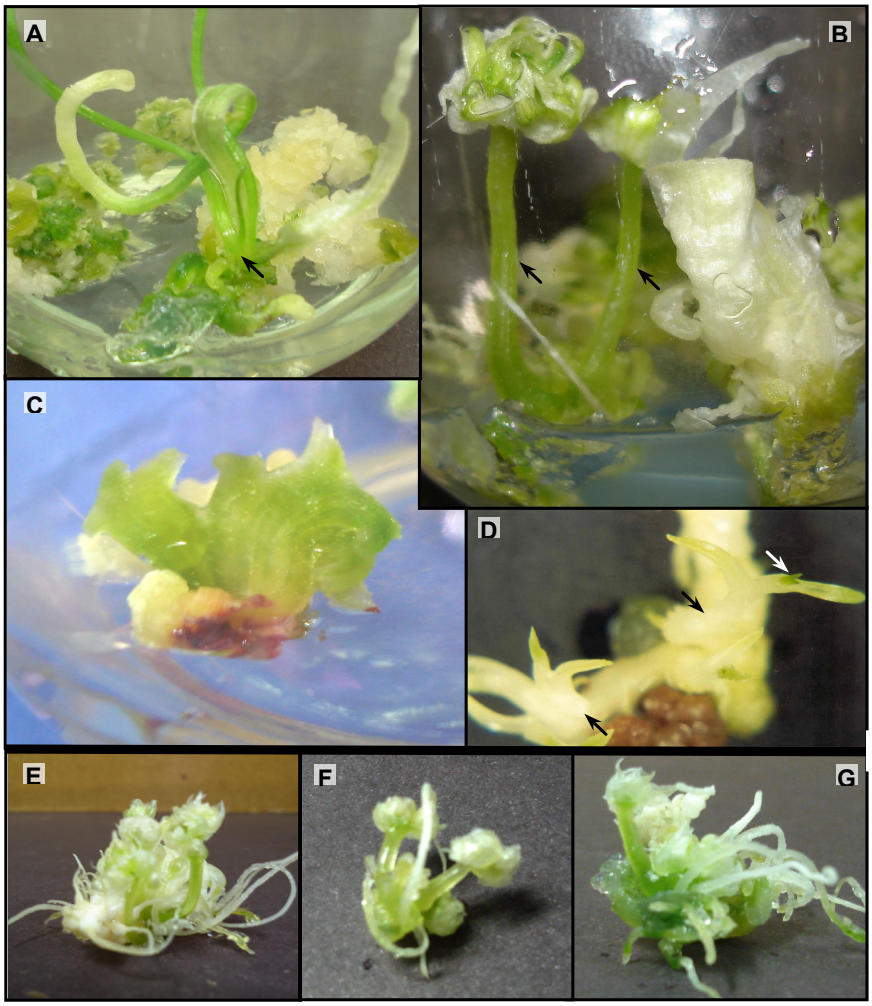

Figure 3. Photos of *in vitro* shoot development of garlic varieties through indirect organogenesis. Regeneration on medium A (Kondo *et al*., 2000) (**A-D**) and medium B (Zheng *et al*., 2003) (**E-G**). (A) Developing shoots from cv. 'Amarante-Embrapa' (arrow); (B) cv. 'Jonas' with two shoots (arrows); (C) cv. 'Cajuru 2315' with initial shoot development; (D) Leaf primordia developing from calli of cv. 'Jonas' callus; (E-G) Developing roots and aerial organs from calli of: (E) cv. 'IAC 75-Gigante de Curitibanos'; (F) cv. 'IAC 63-Mexicano Br' and (G) cv. 'Amarante-Embrapa'. Piracicaba, CENA/USP, 2011.

Agrobacterium tumefaciens.

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