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Comparison of Economic Efficiency between *in Vitro* and Field Methods for Vegetative Propagation of *Coffea Canephora*

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ABSTRACT

Background: *Coffea canephora* is a rustic species of coffee which is drought tolerant and resistant to diseases that commonly affect *C. arabica*. It contributes to about 35% of the world coffee production and is advantageous for the soluble coffee industry. Propagation of *C. canephora* by seeds is undesirable because this method results in high heterozygosity and great genetic variability among populations. Its vegetative propagation is an alternative to avoid this issue and has been successfully achieved by both *in vitro* and field methods, mainly by somatic embryogenesis and rooting of cuttings, respectively. **Objective:** The objective of this study was to approach the viability of the two forms of propagation, comparing cost and time for the production of new plantlets and number of plantlets produced in each propagation system. **Results:** The final cost of a plantlet produced under *in vitro* conditions is US\$ 0.23, while under field conditions is US\$ 0.12, human resources being the highest cost in both systems. The whole *in vitro* process takes 465 days, in comparison to 345 days taken in the field procedures, the acclimatization of plantlets being the lengthiest activity of the *in vitro* process. However, a single plant gives origin to 20,131.8 plantlets via the *in vitro* system, and only 180.2 plantlets via the field system. **Conclusion:** The propagation of *C. canephora* by somatic embryogenesis is more expensive and takes more time than the propagation by rooting of cuttings, despite the fact that the former allows the production of many more plantlets per matrix plant. *In vitro* procedures may be more efficient only when the number of matrix plants is restrictive, as in the case of the launch of new cultivars.

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INTRODUCTION

Coffee is the second most traded world commodity after oil, and one crucial to the economies of several countries, accounting for the bulk of export earnings in more than 50 developing countries of Africa, Asia and Latin America (Bigirimana *et al.*, 2013; Romero *et al.*, 2013). *Coffea arabica* and *C. canephora* are the most cultivated species among more than 100 species of the genus, accounting for the majority of coffee consumed around the world, the latter contributing to about 35% of the world coffee production (Esquivel and Jiménez, 2012; Musoli *et al.*, 2012). *C. canephora* provides the main source of disease and pest resistance traits not found in *C. arabica*, including coffee leaf rust (*Hemileia vastatrix*), Coffee Berry Disease (*Colletotrichum kahawae*) and root-knot nematode (*Meloidogyne* spp.) (Philippe *et al.*, 2009) and because of this is being used in

breeding programs, through which interspecific hybrids between *C. arabica* and *C. canephora* have been successfully produced (Lashermes *et al.*, 2011; Prescott-Allen and Prescott-Allen, 2013). In addition, its deep rooting system, stomatal control of transpiration, leaf area maintenance, and biochemical traits provide drought tolerance, which is especially important in areas where water limitations to coffee production are expected to become increasingly restrictive because of global climate changes (Silva *et al.*, 2013). In relation to sensory properties, *C. canephora* is considered to be inferior and, therefore, commands lower prices on the international market (Esquivel and Jiménez, 2012). However, despite the poorer sensory qualities of *C. canephora*, it has the advantage of allowing extraction of larger amounts of soluble solids, which enables its use in blends and in the soluble coffee industry (Vignoli *et al.*, 2011).

C. canephora reproduces by outcrossing due to its gametophytic self-incompatibility. Consequently,

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propagation by seeds results in high heterozygosity and great genetic variability among populations of this species (Verdin Filho *et al.*, 2014). Therefore, the vegetative propagation is an alternative to avoid variability and has been successfully achieved for propagation of the species. Among the available *in vitro* techniques, somatic embryogenesis is the most utilized for *C. canephora* propagation, for it allows regeneration of numerous plants from small plant tissues or organs and can be used for large-scale clonal propagation of elite cultivars, providing an alternative approach to conventional micropropagation (Arnold, 2008; Deo *et al.*, 2010; Santos *et al.*, 2010). Regarding the propagation of this species under field conditions, the rooting of cuttings is the most commonly used method, because it enables the maintenance of the genetic characteristics of parental plants, ensuring higher uniformity of the crops, among other desirable characteristics, besides the high number of nodal segments produced by a single plant (Santos *et al.*, 2013; Verdin Filho *et al.*, 2014). These systems of propagation of *C. canephora* are current routine protocols and have been practiced and improved at the Brazilian Agricultural Research Corporation (Embrapa) and other Brazilian institutions for a number of years (Fonseca *et al.*, 2007; Santos *et al.*, 2010).

This study presents a comparison between the two methods of propagation of *C. canephora*, taking into account cost and time for the production of new plantlets and number of plantlets produced per matrix plant. In general, there is a lack of studies comparing cost and viability of *in vitro* and field propagation systems, and there is no such study specific to *C. canephora*. This study can also help entrepreneurs in making decisions on the plant production system best suited to their needs and qualifications.

MATERIAL AND METHODS

The values established for the production of plantlets by somatic embryogenesis at the Laboratory of Plant Tissue Culture of Embrapa Rondônia (Porto Velho, Brazil) and by rooting of cuttings at the Experimental Field of the same institution were used as a base for the comparison of *in vitro* and field systems.

Plant material. Leaves and cuttings used in both systems come from *C. canephora* cv. BRS Ouro Preto plants, composed of 15 genotypes. Adult matrix plants (2 y.o.) have their orthotropic branches bent until they touch the ground to stimulate the emission of new orthotropic buds. Six months later these buds are converted into shoots from which leaves and cuttings are taken for the *in vitro* and field procedures, respectively (Fonseca *et al.*, 2007).

***In vitro* propagation system.** Leaves collected from plantlets are washed with water and a detergent agent and immersed in 70% alcohol for 1 minute,

NaOCl 1.25% for 30 minutes and rinsed three times in sterile distilled water. Under aseptic conditions each leaf is cut to produce 20 fragments of about 1.0 cm² which are individually inoculated with the abaxial surface facing upwards in test tubes containing 10.0 mL of medium. The explants are kept in a Primary Culture Medium (PCM) for 60 days to produce somatic embryos in an average of 14.5 embryos per explant. The somatic embryos are transferred to flasks containing 30 mL of a Germination and Maturation Medium (GMM) where they grow and give rise to cotyledons in the following 120 days. After that, the embryos are vertically inoculated in flasks containing 30 mL of a Growth and Rooting Medium (GRM) where they are kept for 150 days to produce leaves and roots. Media composition: *PCM* - half salt concentration of MS medium (Murashige and Skoog, 1962), 10 mg L⁻¹ thiamin, 1 mg L⁻¹ pyridoxine, 1 mg L⁻¹ nicotinic acid, 1 mg L⁻¹ glicyn, 100 mg L⁻¹ inositol, 100 mg L⁻¹ casein, 400 mg L⁻¹ malt extract, 20 g L⁻¹ sucrose, 8 g L⁻¹ agar, 4.92 µM indole-3-butyric acid (IBA), 4.92 µM 6-(gamma,gamma-Dimethylallylamino)purine (2iP), and 20 µM 2,4-dichlorophenoxyacetic acid (2,4-D) (Santos *et al.*, 2013). *GMM* - same composition of *PCM*, without 2iP and 2,4-D, with 4.44 µM 6-Benzylaminopurine (BA). *GRM* - same composition of *GMM*, without casein and malt extract. The media have the pH adjusted to 5.8 before autoclaving (120°C for 20 minutes). *PCM* and *GMM* cultures are kept in darkness; *GRM* culture is kept under 50 µmol.m⁻².s⁻¹ photosynthetic photon flux density light provided by cool white fluorescent tubes, with a 16 h.d⁻¹ photoperiod, at 24±2°C. The plantlets (3-8 cm length) produced are washed in tap water to eliminate residual culture medium and planted in polypropylene trays containing commercial substrate Plantmax®, under nursery controlled conditions of 50% shading, sprinkler irrigation for 15 minutes six times a day, and temperature of 22-32°C, for 120 days, after which the plantlets have 6 pairs of leaves and are ready to be cultivated under field conditions.

Field propagation system. Aiming to produce cuttings, the basal and apical portions of plantlets, the plagiotropic branches, and 2/3 of the leaf blade are eliminated. The cuttings are then individualized by bevel cuts, one 1.0 cm above the insertion of the plagiotropic branches and the other 4.5 cm below the insertion of the pair of leaves. The cuttings are immersed into a fungicide solution (2.5 g L⁻¹ Cuprozeb, 12 g L⁻¹ Mancozeb, 1 g L⁻¹ Penicrom) and then planted at a depth of 2.0 cm in bags with substrate: 75% (v/v) soil horizon (depth 10 cm), 25% (v/v) cattle manure, 0.06% (v/v) dolomitic limestone, 0.48% (v/v) superphosphate, 0.03% (v/v) potassium chloride, 0.012% FTE (fritted trace elements) (Marcolan *et al.*, 2009). From the 50th day of cultivation in intervals of 30 days nitrogen fertilization is provided by a solution of 33.75 mg.L⁻¹

urea (12.5 mL per plant). The bags are kept in a plant nursery where 90% humidity is provided by sprinkler irrigation for 10 seconds every 10 minutes. After 30 days the period between irrigations is 20 minutes. In the first 80 days, the shading is 50%; thereafter is 25%. After 150 days the plants have 6 pairs of completely expanded leaves and are ready to be cultivated under field conditions.

Estimation of costs. The production outcomes were estimated for the effective production of 300,000 plants by each propagation system taking into account 20% of losses on somatic embryogenesis upon the conversion of embryos into plantlets and during the acclimatization process and 10% of losses on field procedures upon the conversion of cuttings into plantlets. *In vitro* propagation costs are comprised of media components and growth regulators, depreciation of equipment, reposition of laboratory material, electric energy, water, salaries of a manager and four employees, and return on capital (opportunity cost), for 345 days of operation of the laboratory; and the costs relative to acclimatization of the plantlets, including substrate, agrochemicals, greenhouse depreciation, reposition of plastic trays and conical plastic containers, electric energy, water, payment of a manager and day laborers, and return on capital relative to 120 days. The costs for propagation by cuttings took into account polyethylene bags, substrate components, fungicides, insecticides, herbicides, depreciation of nursery, irrigation system and tools, reposition of material, electric energy and water, payment of day laborers to produce cuttings, to establish and maintain plantlet cultivation, and return on capital, for 345 days of operation. The values were converted from Real (Brazilian currency) to American Dollar on 28th January 2015 at a rate of US\$1.00 to R\$2.59.

Results:

Throughout the somatic embryogenesis process 15 matrix plants are needed to provide a total of 301,977 acclimatized plantlets ready to be taken to field conditions (Table 1). Each matrix plant gives rise to 50 shoots by the method of bending the orthotropic branches. Each shoot provides two leaves, for only the second pair is used. From each leaf are produced 20 explants, each one giving origin to 14.5 somatic embryos. There is a loss of 22% on the conversion of these embryos, which fail to convert into plantlets, and of 11% during the acclimatization of the plantlets. These losses represent more than 30% of the total number of somatic embryos produced. From one single matrix plant 20,131.8 acclimatized plantlets are produced.

In the system of rooting of cuttings, 1,665 matrix plants are need to produce 300,000 acclimatized plantlets (Table 2). Each matrix plant gives rise to 50 shoots and each shoot is divided into four cuttings. During the conversion of these cuttings into plantlets,

there is a loss of 10%. One matrix plant originates 180.2 acclimatized plantlets.

The cost of production of 300,000 plantlets by somatic embryogenesis procedures is US\$ 68,553.41 (Table 3), the cost of a single plantlet being US\$0.23. The acclimatization of the plantlets accounts for 32.94% of the total cost of production. The highest cost, in both phases, i.e., production of somatic embryos and acclimatization, is the payment of workers, accounting for 53.52 and 18.61% of the total production cost, respectively. The proportion of cost of media components, including growth regulators, is 0.92% of the total cost of production. Under field conditions, to produce 300,000 plantlets by rooting of cuttings costs US\$ 36,334.71 (Table 4) the cost of a single plantlet being US\$0.12. The highest cost is the payment of workers, which represents 50.49% of the total cost, followed by depreciation of nursery, which accounts for 16.17%.

In relation to the time of production, it takes 465 days to produce 300,000 plantlets by somatic embryogenesis (Table 5). The longer phase is the conversion of the embryos into plantlets, which takes 150 days, 32.26% of the whole process, and acclimatization takes 120 days, 25.81% of the total time.

To produce 300,000 plantlets by rooting of cuttings takes 345 days (Table 6), from which 52.17% is taken to produce shoots from matrix plants.

Discussion:

The comparison between Tables 1 and 2 evidences that the initial numbers of matrix plants necessary to produce 300,000 plantlets in the two systems are extremely different. Somatic embryogenesis requires only 15 matrix plants, while rooting of cuttings needs 1,665 plants, which presupposes a large cultivation only to sustain such a propagation system. From one single matrix plant it is possible to produce 20,131.8 plantlets by somatic embryogenesis, while only 180.2 plantlets can be produced from one matrix plant by rooting of cuttings, which evidences the efficiency of the *in vitro* system. Besides, on certain occasions a high supply of matrix plants cannot be available, for example when a new cultivar is launched and there is a limited number of plants to provide propagation by cuttings. In such a situation, somatic embryogenesis turns out to be a reliable and efficient system of propagation, for the greatest agricultural interest in, and the most commercially attractive employment of somatic embryogenesis is its practical application for large-scale vegetative propagation, providing an alternative approach to conventional micropropagation of elite cultivars (Jiménez, 2001; Arnold, 2008; Deo *et al.*, 2010).

In the present *in vitro* protocol, each leaf explant produces 14.5 embryos, which result in a total of 11.3 plantlets (22% lost during conversion). This

result is similar to the study of Giridhar *et al.* (2004), where *C. canephora* leaf explants cultivated on medium supplemented with IAA and BA produced 13.0 embryos each, resulting in 11.1 plantlets (losses of 15%). Almeida *et al.* (2014) studied the embryogenic potential of three genotypes of *C. arabica* and found 68.8, 71.9 and 20.0 embryos per explant.

The field procedures used in the present study result in 90.1% conversion of cuttings into plantlets, as an outcome of successful rooting. Apparently, rooting capacity is highly dependent on the genotype in this species. Fadelli and Sera (2002) found 66.7 and 91.7% rooting in cuttings of two populations of *C. canephora* var. Robusta; Bergo and Mendes (2000) found 97.2% rooting in *C. canephora* cv. Conilon. *C. arabica* is also variable in relation to this aspect, in which rooting percentages range from 20 to 99%, depending on the method and cultivar used (Jesus *et al.*, 2010).

Comparing the costs of the two propagation systems, it is remarkable that the cost of a plantlet produced under field conditions is about half the cost of a plantlet obtained by tissue culture methods (Tables 3 and 4). According to Verdin Filho *et al.* (2014), among the forms of vegetative propagation, micropropagation is a fast and efficient technique that can be used to multiply plants of coffee, however, it is expensive in relation to other multiplication techniques, since it demands specialized laboratories, expensive consumables and trained workers. The cost of production of a plantlet by somatic embryogenesis is US\$ 0.23. Almost 75% of this cost refers to the payment of human resources to produce the embryos and to acclimatize the plantlets. The manager must have a certain level of expertise in tissue culture procedures to run the production, which can considerably increase the expenses. It is notable that the cost of acclimatization represents about one third of the total cost, while the culture media cost accounts for less than 1% of the whole process. Carvalho *et al.* (2013) studied the costs of production of 400,000 plantlets of *C. arabica* by somatic embryogenesis and observed quite different results; a plantlet cost is US\$ 0.37, value almost 70% higher than the cost per plant obtained by this approach, probably because the authors evaluated a more expensive system, involving maturation of embryos in bioreactors and due to the higher cost of the acclimatization, more than double the acclimatization cost of *C. canephora* plants. This species is remarkably rustic in relation to *C. arabica* (Santos *et al.*, 2013) demanding less sophisticated acclimatization procedures, less time and consequently lower costs. Besides, according to Pereira *et al.* (2007) there is a great difference between these two coffee species in relation to the induction of direct somatic embryogenesis in leaf explants. *C. canephora* unleashes the direct formation of somatic embryos more easily and

rapidly than *C. arabica*, whose somatic embryos present lower levels of development. The cost of production of a plantlet by rooting of cuttings is US\$ 0.12. As well as with the *in vitro* propagation, the highest cost is the payment of workers, reaching half of the total cost of production.

The only study in the literature regarding cost of production of coffee plantlets *in vitro* is that previously cited carried out by Carvalho *et al.* (2013), who evaluated the production cost of 400,000 plantlets of *C. arabica* by somatic embryogenesis. There are few studies comparing the production of plants under field and *in vitro* conditions. Khaled *et al.* (2010) studied the production of banana plants and estimated that the total cost of production of plants by tissue culture techniques exceed by 29.8% the traditional method of propagation. However, the authors recommend tissue culture for banana propagation, considering that the plants produced by *in vitro* techniques have higher quality in relation to those produced by conventional methods and are free of viral diseases. Mng'omba *et al.* (2008) compared the viability of several methods for propagation of fruit trees and noted that the equipment, expertise and relative cost of *in vitro* techniques are very high and thus considered micropropagation generally as the last option in the selection of a propagation method. Nevertheless, the authors emphasize the importance of micropropagation in breeding programs, for virus elimination, or to widen the cultivation of a new cultivar with a high market price.

Regarding the periods of time required to produce plantlets by both propagation systems (Tables 5 and 6), it is noticeable that the *in vitro* system takes 465 days, much longer than the field system, which takes 345 days, with a difference of 120 days, the exact period of time required to acclimatize the plantlets in the somatic embryogenesis system. *In vitro* produced plantlets are more fragile and so need time to be adapted to outdoor conditions, during the period of transition from the mixotrophic condition (*in vitro* plantlets are not fully dependent on their own photosynthesis) to the autotrophic condition (George and Debergh, 2008). Santos *et al.* (2014) studied the acclimatization of *C. canephora* plantlets and concluded that this is a vulnerable phase in which the most critical factor is the availability of water for the plantlets. In the field procedures, the most relevant activity in terms of time is the induction of shoots from matrix plants, which takes 180 days, time necessary for the orthotropic branches to produce buds and for their growth into orthotropic shoots (Fonseca *et al.*, 2007).

The somatic embryogenesis system utilized by Carvalho *et al.* (2013) to produce 400,000 plantlets of *C. arabica* takes 660 to 720 days, 42 to 55% more time than the system used in this approach to *C. canephora* propagation. This difference is due to the

inherent characteristics of the species, as *C. arabica* is more vulnerable to desiccation. Vieira *et al.* (2013) remark that *C. canephora* species have strategies to cope with drought, such as leaf folding and inclination, which reduce leaf abscission. Besides, the authors mention differences in root depth, plant-hydraulic conductance and stomatal control of water use as characteristics of tolerant genotypes of this species.

Conclusion:

Somatic embryogenesis requires more time and money than the rooting of cuttings to propagate *Coffea canephora* plants, despite the fact that the later requires many more matrix plants to initiate the production system. Therefore, it is remarkable that somatic embryogenesis is more efficient as a propagation system of this species only when the number of matrix plants is limited, as in the case of the launch of new cultivars.

Table 1: Number of *C. canephora* plant structures and ratio of multiplication throughout somatic embryogenesis.

Number of plant structures	Ratio of multiplication
15 matrix plants provide 750 shoots	1 : 50
750 shoots provide 1,500 leaves	1 : 2
1,500 leaves provide 30,000 explants	1 : 20
30,000 explants produce 435,000 somatic embryos	1 : 14.5
435,000 embryos produce 339,300 plantlets	1 : 0.78
339,300 plantlets produce 301,977 acclimatized plantlets	1 : 0.89
General ratio	1 : 20,131.8

Table 2: Number of *C. canephora* plant structures and ratio of multiplication throughout rooting of cuttings.

Number of plant structures	Ratio of multiplication
1,665 matrix plants produce 83,250 shoots	1 : 50
83,250 shoots produce 333,000 cuttings	1 : 4
333,000 cuttings produce 300,000 plantlets	1 : 0.9
General ratio	1 : 180.2

Table 3: Production costs of 300,000 plantlets of *C. canephora* by somatic embryogenesis.

Expense	Total cost (USD)	Unit cost (USD)	Percentage of the total cost (%)
Production of embryos	45,974.22	0.153247	67.063
PC medium	37.22	0.000124	0.054
GM medium	99.27	0.000331	0.144
GR medium	497.21	0.001657	0.725
Equipment depreciation	2,463.81	0.008213	3.594
Material reposition	1,541.66	0.005139	2.249
Electric energy	1,907.17	0.006357	2.782
Water	140.28	0.000468	0.205
Salary of manager	12,228.99	0.040763	17.839
Salary of employees (4)	24,457.99	0.081527	35.677
Return on capital	2,600.62	0.008669	3.794
Acclimatization of plantlets		0.075264	32.937
	22,579.19		
Substrate	2,095.84	0.006986	3.057
Agrochemicals	423.39	0.001411	0.618
Greenhouse depreciation	3,518.28	0.011728	5.132
Reposition of material	1,539.59	0.005132	2.246
Electric energy	402.20	0.001341	0.587
Water	561.14	0.001870	0.819
Salary of manager	4,253.56	0.014179	6.205
Wage of day laborers	8,507.12	0.028357	12.409
Return on capital	1,278.07	0.004260	1.864
Total	68,553.41	0.228511	100.000

Table 4: Production costs of 300,000 plantlets of *C. canephora* by rooting of cuttings.

Expense	Total cost (USD)	Unit cost (USD)	Percentage of the total cost (%)
Polyethylene bags	2,581.76	0.008606	7.105
Substrate	4,129.87	0.013766	11.366
Agrochemicals	1,329.04	0.004430	3.658
Nursery depreciation	5,874.05	0.019580	16.167
Electric energy	136.35	0.000455	0.375
Water	1,613.27	0.005378	4.440
Salary of manager	6,114.50	0.020382	16.828
Wage of day laborers	12,228.99	0.040763	33.657
Return on capital	2,056.68	0.006856	5.660
Total	36,334.71	0.121116	100.000

Table 5: Period of time required to produce 300,000 plantlets of *C. canephora* by somatic embryogenesis.

Activity	Period of time (days)	Percentage of the total time (%)
Collection and disinfection of leaves from shoots	15	3.23
Production of somatic embryos on PC medium	60	12.90
Maturation of somatic embryos on GM medium	120	25.81
Conversion of embryos into plantlets on GR medium	150	32.26
Acclimatization of plantlets	120	25.81
Total	465	100.00

Table 6: Period of time required to produce 300,000 plantlets of *C. canephora* by rooting of cuttings.

Activity	Period of time (days)	Percentage of the total time (%)
Induction of shoots from matrix plants	180	52.17
Production of cuttings from shoots	15	4.35
Formation of plantlets from cuttings	150	43.48
Total	345	100.00

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