Dilution of nutrient elements formulation in culture media for in vitro conservation of *Coffea arabica* AS2K variety

M S D Ibrahim*, E Randriani and N Ajijah

Senior researcher, Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), IAARD, Jalan Raya Pakuwon Km 2 Parungkuda, Sukabumi 43357 Indonesia

*Email: meynartisaya@yahoo.com

Abstract. In vitro conservation technology is one of the strategies to maintain the genetic diversity of perennial plants, such as Arabica coffee. The purpose of this study was to examine the effect of nutrient elements dilution on the emphasis of growth of Arabica coffee microcuttings of AS2K variety. The plant materials were Arabica coffee plantlets of AS2K variety which were reproduced using Murashige and Skoog (MS) media and given BA 2 g L\(^{-1}\), sucrose 30 g L\(^{-1}\), and phytagel 2.5 g L\(^{-1}\). The treatment conservation used MS medium without adding growth regulator with sucrose 30 g L\(^{-1}\) and phytagel 2.5 g L\(^{-1}\). The examined treatment was the dilution of MS nutrient elements into a half (1/2) and a quarter (1/4) of the basic concentration, the full (basic) MS as the control. The experimental design used was a completely randomized design with 10 replications. The results showed that Arabica coffee culture on 1/2 MS media can be considered for conservation since it is cost-effective, able to reduce the growth of shoots and produce more rooted plantlets than controls. This media culture can be stored for 8 months without morphological changes so that it can recover spontaneously.

1. Introduction
Conservation of genetic diversity of Arabica coffee (*Coffea arabica* L.) contributes to provide a source of genetic information needed for breeding programs to generate coffee plants with better quality. One of the main problems encountered in Arabica coffee development is conserving its genetic diversity. Conservation of Arabica coffee plants is generally undertaken in situ. However, this method is considered less effective, because of the threats to the genetic resources of Arabica coffee plants in the field such as crop loss due to aging, improper agricultural practice, pests and diseases attack, and global climate change that are essential for Arabica coffee’s growth are no longer suitable. Arabica coffee plants require altitude, shade, and humidity relatively high [1]. Ecosystem and natural environmental changes due to natural disasters such as floods, earthquakes, drought, fires, and volcanic eruptions, are also some obstacles to in situ conservation [2,3]. Additionally, in situ conservation also requires greater land areas, high operating costs, and complicated management [4].

Therefore, ex situ conservation is a feasible way to save plants from extinction. Ex situ conservation can be carried out by maintaining plant genetic resources under controlled conditions in botanical gardens, nurseries, and seed storage or gene banks through in vitro conservation [5,6].

In vitro conservation using active collections is usually carried out using plants in normal growth and formulations media for propagation. This active collection usually lasts only 2-3 months and due to the decreasing nutrients hence requires another subculture for new media [7], making it less effective since
it requires a high frequency of subcultures. Conservation in vitro Coffea spp in this way has been reported by Bertand-Desbrunains et al [8].

Several in vitro germplasm conservation strategies have been developed to minimize the frequency of subcultures, including conservation by growth reduction or miniaturization of suspending or stop the growth [9]. The slow or minimal growth preservation can be done by manipulating physical factors such as low-temperature storage, low light intensity and shoot photoperiod, application of ethylene inhibitors and small size of culture vessels, and chemical factors such as minimal media, used plant growth regulator, and osmotic potential preservation [10].

The use of cryopreservation methods can extend the storage period so that it is suitable for long-term plant conservation [11]. Cryopreservation using Coffea conophora seeds was reported by Coelho et al [12]. The main difficulty with the cryopreservation method is that the procedure is very technical and expensive because it involves a large number of resources and labor. The use of retardants in culture media has also been used in in vitro conservation of Musa acuminata x balbisiana Colla [13], Pimpinella pruatian Molk. [14], Zingiber purpureum Roxb [15] and Coffea arabica L [16], but it may cause residual effects hence it requires a recovery phase before it can grow back to normal.

Reducing nutrients or carbon is one of the conservation methods that does not require high costs. Cultures that were undergone such treatment showed no decrease in growth nor morphological changes in character and anatomical. In vitro conservation of coffee by reduced concentration sucrose and low temperature has been done [17]. The results indicated that there are differences in shelf life in the coffee species studied. This study aimed to examine the effect of nutrient elements dilution on the emphasis of growth of Arabica coffee micro cuttings of AS2K variety.

2. Methods
The study was conducted at the Tissue Culture Laboratory, Indonesian Industrial and Beverage Crops Research Institute (IIBCRI), from July 2017 to December 2018. The planting materials used were the Arabica coffee plantlets of AS2K variety, collection of tissue culture laboratories at IIBCRI. The activities included: (1) Propagation of in vitro Arabica coffee shoots as explants source, (2) In vitro shoots conservation of Arabica coffee on various nutrient element formulation (full, half, and a quarter) and shoot multiplication of Arabica coffee after in vitro conservation.

2.1. Propagation of in vitro shoots Arabica coffee as explant source
The Arabica coffee shoots were propagated using Murashige and Skoog (MS) medium with growth regulators BA 2 mg L\(^{-1}\), 30 g L\(^{-1}\) sucrose, and phytagel 2.5 mg L\(^{-1}\) [18]. The incubation conditions for propagation were room temperature at 23 ± 2 °C, irradiation intensity of 800 - 1000 lux with 16 hours of bright photoperiodicity. The study was conducted until sufficient explants obtained for further activities.

2.2. In vitro shoots conservation of Arabica coffee on various nutrient element formulation (full, half, and a quarter) and shoot multiplication of Arabica coffee after in vitro conservation
The Arabica coffee plantlets were cut into one segment. Apical cuttings were then planted onto the treatment media (full MS, ½ MS, and ¼ MS) which were given 30 g L\(^{-1}\) sucrose and 2.5 g L\(^{-1}\) phytagel. The pH of the medium was adjusted to 5.6 ± 0.1. Media were sterilized using an autoclave for 15 minutes at 121°C. The incubation condition for in vitro conservation was in a room at 23 ± 2°C, the irradiation intensity of 800 - 1000 lux with 16 hours of bright photoperiodicity. After the conservation period, viable cultures from each treatment were transferred into multiplication media for storage. Growth regulators of 2 mg L\(^{-1}\) BA, 30 g L\(^{-1}\) sucrose, and 2.5 mg L\(^{-1}\) phytagel were added into multiplication media.

2.3 Data analysis
The experimental design was arranged factorially in a completely randomized design (CRD) with 10 replications. Each replication consisted of 2 micro cuttings, so that the total micro cuttings in one
treatment was 20 micro cuttings. The observed variables were the number of growing cuttings, the number of leaves formed, the number of fresh leaves, the number of wilted leaves, and the number of roots. The variables were observed every 2 months until plantlets could not grow. The collected data were analysed using analysis of variance (ANOVA), if significantly different followed by Duncan Multiple Range Test (DMRT) at a level of 5%.

3. Results and Discussion
3.1. Propagation of in vitro shoots of Arabica coffee as explant source
The Arabica coffee explants had a high proliferation rate in propagation media. Each shoot in the media could produce on average 1.30 shoots from apical buds and 3.9 shoots from axillary buds. The shoots and leaves appear fresh. Such culture conditions were ideal to be used as a source of explants. The number of shoots generated in the propagation media was shown similarly with previous studies, 4.0 micro shoots [18].

3.2. In vitro shoots conservation of Arabica coffee on various nutrient element formulation (full, half, and a quarter) and shoot multiplication of Arabica coffee after in vitro conservation
The shoot length of Arabica coffee plantlets
The results of 8 months study showed that the shoot length of Arabica coffee plantlets increased with conservation time. In this study, accelerated growth of cultures took place from the 4th to the 6th month, followed by stagnation (Figure 1). The shoot length increased rapidly after four months as the plantlet is undergoing cell division (increase in number) and cell enlargement (increase in size) very quickly. The performance of Arabica coffee growth at all treatment media from two to eight month can be seen in Figure 2. The colour of culture media changed from clear white to yellow with conservation time. This is because the long conservation period causes phenol compounds to accumulate in the culture media. The solid culture medium becomes liquid indicates that the strength of phytagel has decreased.

![Figure 1](chart.png)

**Figure 1.** The average shoots length of *Coffea arabica* plantlets in treatment media with various nutrient element formulations at 2, 4, 6, and 8 months after culture. The same letters on top of the bar indicate the treatment was not significantly different at Duncan $\alpha = 0.05$. 
Figure 2. A-D. Growth of Arabica coffee plantlets in the full MS medium (A. Two months. B. Four months. C. Six months and D. Eight months after culture). E-H. Growth of Arabica coffee plantlets in the ½ MS treatment medium (E. Two months. F. Four months. G. Six months and H. Eight months after culture). I-L. Growth of Arabica coffee plantlets in the 1/4 MS treatment medium (I. Two months. J. Four months. K. Six months and L. Eight months after culture).

The growth of Arabica coffee plantlets also has a special pattern of growth within one growing cycle that similar to other plantlets. The pattern was fast growth at the vegetative phase until a certain point then slows down due to plant cell growth and finally decreases in the senescence phase. In this study, plantlet height experienced a phase of stagnation during the 6th month after conservation. This was allegedly due to reduced nutrients in media culture in the 6th month, so that plantlet growth has decreased. It was similar to the plantlets or shoots that were transferred to media the conservation media [19].

The number of leaves formed
The number of leaves formed in this study increased along with the culture period, except for ¼ MS treatment. In the media ¼ MS, the increase in the number of leaves at 4 months after culture tends to be stagnant. Statistical analysis showed that in two months after culture, the average number of leaves formed was not significantly different between treatments. The treatment was significantly different at 4 months after culture. The full MS and ½ MS treatments were significantly different from the MS for the number parameter (Figure 3).
Figure 3. The average number of leaves of *Coffea arabica* plantlets in treatment media with various nutrient element formulations at 2, 4, 6, and 8 months after culture. The same letters on top of the bar indicate the treatment was not significantly different at Duncan $\alpha = 0.05$.

The number of leaves had a positive correlation with the concentrations in culture media decreasing. However, the decrease of concentration $\frac{1}{2}$ MS did not differ significantly from the full MS formed. The results of this study was in line with the results of research conducted by Castilla-Valdes *et al* [20] where the number of leaves of Arabica coffee plantlets formed between full MS and $\frac{1}{2}$ MS was not significantly different.

The number of senescence leaves

The number of Arabica coffee senescence leaves were not found in 2 and 4 months after culture (MAC). Senescence leaves began to be found in 6 MAC, and increased in 8 MAC. The number of leaves with the highest senescence was found in $\frac{1}{4}$ MS treatment and significantly different from full MS and $\frac{1}{2}$ MS (Figure 4). This senescence process indicates that the nutrient macro and micro-content in the culture media has begun to decrease so that plantlets cannot obtain nutrients. Before wilting, the leaves appeared to change colour from green to yellowish, yellow, and brown. This discoloration occurs due to physiological and biochemical processes that begin when photosynthesis stops, chlorophyll degradation occurs, anthocyanin pigment accumulation, decreased starch content, reduced RNA and protein, DNA molecules degraded by DNase enzymes, decreased cytokinin content and, increased deteriorative hormone content [21].

The senescence process is a physiological strategy of the plants to conserve nutrients. As it can be seen in this study, the process of senescence of Arabica coffee leaves has begun to run in the culture since 6 months after culture in all treatments, with the most number in the treatment $\frac{1}{4}$ MS. This result was somewhat different from the research of Castilla-Valdes *et al* [20] which shows that the process of senescence of Arabica coffee leaves occurs 4 months after culture. This difference was allegedly due to differences in the type of coffee used.
Figure 4. The average of senescence leaves *Coffea arabica* in treatment media with various nutrient element formulations at 2, 4, 6, and 8 months after culture. The same letters on top of the bar indicate the treatment was not significantly different at Duncan $\alpha = 0.05$.

The percentage of living plantlets

Studies on *in vitro* conservation, the storage period, and the percentage of surviving explants during storage are important. This study showed that the average percentage of living plantlets was 100% in up to the 4 MAC. The number of surviving plantlets began to decrease in MS media culture at 6 MAC and continued to decrease in 8 MAC. There were decrease in 6 and 8 MAC in the $\frac{1}{2}$ MS treatment but it was not significantly different from full MS (Figure 5). The performance of Arabica coffee plantlets stored for 8 months can be seen in Figure 2D, 2H, and 2L.

Figure 5. The average survival percentage of plantlets *Coffea arabica* in treatment media with various nutrient element formulations at 2, 4, 6, and 8 months after culture. The same letters on top of the bar indicate the treatment was not significantly different at Duncan $\alpha = 0.05$. 
The result of this study is different from what has been reported by previous research that stated the decrease of the number of living plantlets occurred in 4 MAC and there were significant differences between the full MS, ½ MS, and the treatment of ¼ MS [20]. This difference was possibly due to the different genotypes of Arabica coffee used. As demonstrated by Arrigoni-Blank et al. which showed the differences in the response of 4 genotypes of Ipomoea batatas (L.) Lam to nutrient dilution and in vitro concentration, 2 genotypes were significantly different between full and ½ MS while the other 2 genotypes were not significantly different [22].

The Number of roots
Arabica coffee plantlets showed no roots in 2 MAC and 4 MAC. Roots begin to form in 6 MAC and increased in number in 8 MAC. There was no significant difference between the treatments, even though the number of roots in the ½ MS treatment was higher than other treatments (Figure 6). The higher number of Arabica coffee roots in the medium ½ MS compared to ¼ MS was also seen in previous studies [20].

![Figure 6](image)

Figure 6. The average number of roots of Coffea arabica in treatment media with various nutrient element formulation at 2, 4, 6, and 8 months after culture.

Shoot multiplication of Arabica coffee after in vitro conservation
Recovery period after conservation of shoots must be as short as possible for returning cultures to in vitro optimal growth with minimum effort, to be ready for their use in further experiments. The effect of shoots grew after 8 months in conservation medium was tested multiplication. The multiplication of shoots looked good with 3.8 shoots. In this recovery media, the number of shoots produced on 1/4 MS medium was the least compared to other treatments. The performance of Arabica coffee growth from 4 months in medium multiplication is seen in Figure 7.
The storage period is the most important factor in conservation, especially in minimal growth techniques. Effective conservation is conservation that has a relatively long period of time without subcultural action. In this Arabica coffee conservation, the maximum storage period is 8 months.

Based on the results, this study proved that the decrease in nutrient content through media dilution has not been able to extend the shelf life of Arabica coffee culture compared to the full formula. However, these results also indicated a reduction in nutrient content lower than the full formula proved to be enough to sustain the growth of Arabica coffee culture for in vitro conservation. In this case, the use of diluted media can be more cost-effective.

The use of ½ MS media for in vitro conservation of Arabica coffee is not only for efficiency reasons but also for the better productivity of plantlets. Results showed plant height parameters in ½ MS media were lower than full MS, the number of leaves formed and senescence was equal to those of full MS media, as well as the percentage of plantlets. In addition, the number of roots is higher than other treatments.

4. Conclusions
The results show that it is not recommended to reduce the macro and micronutrients in the MS medium formulation into 1/4 as it could decrease the percentage of plantlet survival by more than 50% at 8 months after culture. The plantlet life percentage could reach 100% with the application of ½ MS so that it is more recommended for in vitro culture of Arabica coffee. In addition, this formulation needs fewer chemicals so it can save cost, reduce the growth of shoots and, produces more rooted plantlets than controls. Another advantage of ½ MS media formulation in conservation Arabica coffee is it can be used for 8 months without causing morphological changes so that it can recover spontaneously.

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