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EX VITRO ROOTING OF THREE BLACK PEPPER CULTIVARS

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ABSTRACT: Compared to propagation method via herbaceous cuttings, micropropagation presents higher multiplication rate in less space and time and better phytosanitary control, but with higher costs. The induction of the root system during the acclimatization phase is an alternative to reduce costs in the *in vitro* propagation system. In order to induce roots in the acclimatization phase, the effect of different NAA plant regulator concentrations on the *ex vitro* rooting and acclimatization for the production of black pepper seedlings was evaluated. The experimental design was in randomized blocks, in a 3x4 factorial scheme, with three black pepper cultivars: 'Apra', 'Bragantina' and 'Kuthiravally' and four NAA concentrations: 26.85, 53.70; 80.56; 134.26 µmol/L, six replicates with two plants per plot. The variables evaluated were: survival and rooting rate; roots number per shoot (RN); length of the longest root (LR); number of leaves (NL), root dry matter (RDM) leaf area and and shoot dry matter (LSDM). Data obtained for each variable were submitted to analysis of variance, considering the 3 x 4 factorial model, with three cultivars and four NAA doses, and when significant by the F test, the mean comparison test, the Tukey test ($p < 0.05$), was applied. There was difference for variables NR, LR, NL, LSDM for the source of variation cultivar *versus* NAA doses, demonstrating the presence of interaction between factors and that the NAA effect depends on concentration and cultivar used, that is, the cultivar specifically responds to NAA concentration, being necessary to evaluate the results of interactions. However, for variable RDM, the difference was due to cultivar, with no interaction with NAA concentration. Shoot rooting can be induced during the acclimatization phase as a viable alternative for the three black pepper cultivars. All NAA concentrations promote rooting, but the 'Kuthiravally' cultivar is more sensitive to rhizogenesis, and lower concentrations are recommended to reduce production costs.

KEYWORDS: naphthaleneacetic acid; micropropagation; *Piper nigrum*

ENRAIZAMENTO *EX VITRO* DE TRÊS CULTIVARES DE PIMENTEIRA-DO-REINO

RESUMO: A micropropagação em comparação ao método de propagação via estacas herbáceas apresenta maior taxa de multiplicação em menor espaço e tempo; melhor controle fitossanitário, porém os custos elevados. A indução do sistema radicular durante a fase de aclimatização é uma alternativa para redução dos custos no sistema de propagação *in vitro*. Com o objetivo de induzir raízes nos brotos na fase de aclimatização, avaliou-se o efeito de diferentes concentrações do regulador vegetal, ANA, no enraizamento *ex vitro* e aclimatização para produção de mudas de pimenteira-do-reino. O delineamento experimental foi em blocos ao acaso, em esquema fatorial 3x4, sendo três cultivares: 'Apra', 'Bragantina' e 'Kuthiravally' e quatro concentrações de ANA: 26,85, 53,70; 80,56; 134,26 µmol/L, seis repetições com duas plantas por parcela. As variáveis avaliadas foram: taxa de sobrevivência e enraizamento; número de raízes por broto (NR); comprimento da maior raiz (CR); número de folhas (NF), massa seca da raiz (MSR) e da massa seca da parte área (MSPA). Os dados obtidos, para cada variável, foram submetidos à análise de variância, considerando o modelo fatorial 3 x 4, sendo três cultivares e quatro doses de ANA e quando significativa pelo teste F, foi aplicado o teste de comparação de médias, tukey ($p<0,05$). Houve diferença para as variáveis NR, CR, NF, MSPA para a fonte de variação cultivar *versos* doses de ANA, demonstrando que houve uma interação entre os fatores e que o efeito de ANA depende da concentração e da cultivar utilizada, ou seja, a cultivar responde especificamente a concentração de ANA, sendo necessário proceder o desdobramento das interações. Entretanto, para a variável RDM, a diferença ocorreu devido a cultivar, sem interação com a concentração de ANA. O enraizamento dos brotos pode ser induzido durante a fase de aclimatização como uma alternativa viável para as três cultivares de pimenteira-do-reino. Todas as concentrações de ANA promovem o enraizamento, mas a cultivar Kuthiravally é mais sensível para a rizogênese, e recomenda-se as concentrações mais baixas para reduzir os custos do método de propagação.

PALAVRAS CHAVE: ácido naftalenoacético; micropropagação; *Piper nigrum*

INTRODUCTION

Black pepper, scientifically known as *Piper nigrum* L., is a spice traded worldwide, whose economic use dates back to the early Christian era, sometimes used as an exchange currency, and for a long time was trade product between Europe and Asia (Albuquerque and Condurú, 1971). In Brazil, its cultivation was initially restricted to home-grown orchards in the northern and northeastern regions (Duarte et al., 2002).

Currently, Brazil stands out in the world scenario of black pepper producers, and is among the main producers; and according to the Brazilian Institute of Geography and Statistics – IBGE (2018), the states of Pará and Espírito Santo are the largest producers. The social and economic importance of black pepper is related to the fact that it is mostly a small-producer crop, generating income, fixing labor in the countryside, and being suitable as an alternative crop for family farming (Ribeiro et al., 2019).

The commercial propagation of black pepper occurs vegetatively, where cuttings are removed from orthotropic branches, but they do not always have the appropriate phytosanitary conditions, since some genotypes, even infected with phytopathogens, are asymptomatic (Abbasi et al, 2010; Ramos et al. 2020). *In vitro* propagation has the advantage, compared to the conventional system, of providing higher multiplication rate in less time and phytosanitary control regardless of time of year; however, micropropagation has high cost, both in the implementation and maintenance of the cultivation of plants in an *in vitro* environment (Shahzad, 2017).

The need to have an established protocol for the vegetative propagation of the crop in order to generate viable seedling production on a commercial scale, is reported in literature (Secundino et al., 2014). The induction of the *ex vitro* root system is an alternative to reduce costs and optimize time in the *in vitro* propagation process, unifying the rooting and acclimatization phases (Phulwaria et al., 2013), with the aim of making the micropropagation process economically viable.

Roots *in vitro* produced usually do not survive after the acclimatization stage, requiring the induction of the rooting process through the application of phytoregulators (Oliveira-Cauduro et al., 2016; Shahzad et at., 2017). At the end of the process, with plants showing high survival rates and lower cost, the use of this propagation method becomes viable.

The acclimatization of micropropagated plants is a limiting phase, as seedlings that have grown under totally artificial and controlled conditions (with low light intensity in culture medium containing sucrose and nutrients) pass to new conditions, in which they must adapt to allow heterotrophic growth in the new environment. The leaves of plants cultivated *in vitro* have smaller amount of epicuticular waxes, which, associated with the poor functioning of stomata, make them susceptible to large water losses through transpiration. Thus, this transition from controlled environment to *ex vitro* development is very delicate, so all the material must remain in an environment with indirect sunlight (shade) and high relative humidity (Chandra et al., 2016; Shahzad et at., 2017).

Auxins are commercially used in agriculture for the rooting of cuttings for plant propagation, as occurs in tissue culture at the shoot rooting stage (Carvalho et al., 2011). These play a central role in the rhizogenic development for plant regeneration, whose main effect is linked to the action on root induction and initiation (Saini et al., 2013), such as naphthaleneacetic acid (NAA), a synthetic auxin used in micropropagation to stimulate rhizogenesis.

The *ex vitro* rooting process is carried out directly on the substrate used to obtain seedlings. Therefore, the aim of this work was to evaluate the effect of different NAA plant regulator concentrations on the *ex vitro* rooting and acclimatization of black pepper, aiming at seedling production.

MATERIAL AND METHODS

The work was conducted at the Laboratory of Genetic Resources and Plant Biotechnology and greenhouse at “Embrapa Amazônia Oriental”, Belém-PA. The unification of the rooting stages and acclimatization of black pepper shoots in the micropropagation process was carried out in three cultivars: ‘Apra’, ‘Bragantina‘and ‘Kuthiravally’.

For the experiment installation, apical buds of *in vitro* cultivated shoots were used as explants source for multiplication in MS culture medium (Murashige and Skoog, 1962), added of sucrose 30 g/L, combined with 1.14 μmol/L of IAA (indoleacetic acid) and 2.22 μmol/L of BAP (6-Benzylaminepurine) and solidified with 2 g/L phytagel. The culture medium pH was adjusted to 5.8 before autoclaving at 120 ^{o}C and 1 atm for 20 minutes. Cultivation took place in a growth room under controlled temperature conditions (25 ± 3 ºC), 16-hour photoperiod and 50 μmol m^{-2} s irradiance provided by daylight fluorescent lamps, during a six-week cultivation period. After this *in vitro* multiplication phase, shoots were taken to the washing room, immersed in water to remove excess culture medium and washed in running water before being submitted to NAA solution.

A preliminary experiment (to determine the range of doses that had the best result and thus set up the next experiment) was installed for the selection of NAA doses, in RBD, in a 2x8 factorial scheme, with two cultivars (‘Apra’ and ‘Bragantina ‘), and eight NAA concentrations (0; 5.37; 13.43; 26.85; 134.26; 268.8; 537 and 1074 μmol/L). The base of shoots was beveled and immersed in NAA solution for one minute, then transplanted to vermiculite substrate in polypropylene tray with twenty-four cells, 36x30x9 cm, containing six replicates and two plants per plot. After six weeks, the biometric characteristics were evaluated (shoot height, number of leaves, number and length of roots and shoots submitted to *ex vitro* rooting). The experiment was carried out in greenhouse with temperature of ±28 °C and relative humidity above 90%.

Based on results of this experiment, a new experiment following the same protocol already described was set up, but now with only four NAA concentrations (26.85, 53.70; 80.56; 134.26 μmol/L). The design was in randomized blocks in a 3x4 factorial, with three cultivars (‘Apra’, ‘Bragantina’ and ‘Kuthiravally’) and four NAA concentrations, totaling 12 treatments, each consisting of six replicates and two plants per plot.

In greenhouse, black pepper shoots submitted to solutions with different NAA concentrations remained in trays containing vermiculite, previously moistened, for *ex vitro* rooting and shoot acclimatization. High relative humidity (>90%) was provided by nebulization. Weekly, the vermiculite substrate was moistened with nutrient solution containing half the concentration of MS culture medium salts (½ MS).

After six weeks of cultivation in substrate, plant survival rate and root system growth were evaluated: number of roots (NR), root length (RL), root and shoot dry matter (SDM): number of leaves (NL), leaf area and shoot dry matter (LSDM). The length of the longest root was measured with the aid of digital caliper (accuracy of 0.01 mm). For dry mass analysis, plants were dissected into root and shoot (stem and leaf), which were separately in paper bags and dried in forced ventilation oven at 65 °C until reaching constant dry mass (48 hours), with six replicates, two plants per replicate.

Data obtained for each variable were submitted to analysis of variance, considering the 3 x 4 factorial model, with three cultivars and four NAA doses. When significant difference was identified by the F test ($p<0.05$), the mean comparison test, the Tukey at 5% probability level, was applied. Statistical analyses were performed using the R software (R Core Team, 2016), with the ExpDes.pt package (Ferreira et al. 2014).

RESULTS AND DISCUSSIONS

The previous experiment (exploratory) with two black pepper cultivars (‘Apra’, ‘Bragantina’) allowed the indication of the most adequate NAA doses to evaluate the effect of absence (0 μmol/L) with the lowest root induction rate at maximum dose (1,074 μmol/L) in which short and thick roots were observed. The highest survival rates for the two black pepper genotypes occurred at doses of 26.85 and 134.26 μmol/L, minimum and maximum, respectively. From this, doses to compose the new experiment were selected.

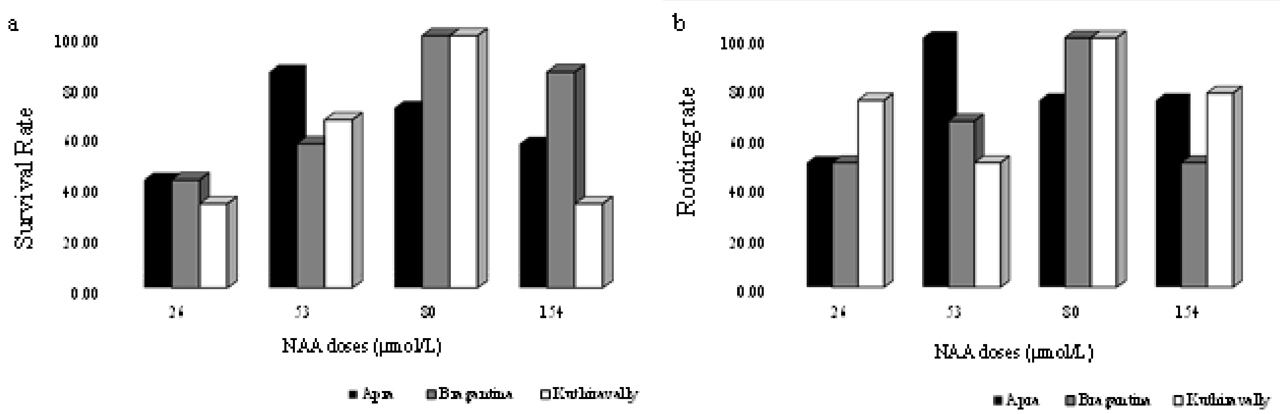
Black pepper shoots, before being submitted to the *ex vitro* rooting process, were evaluated for stem length, number of leaves and buds. For all characteristics evaluated, no statistical difference was observed, characterizing the standardization of shoots submitted to rooting, all with size greater than 1.0 cm (Table 1). According to Souza and Pereira (2007), the standardization of shoots is crucial for the rooting process, associated with the microcutting quality, constituting the size (≥1.0 cm) and the developed leaf system, which is fundamental for the production of auxins, nutrients, sucrose or nitrogen compounds necessary for root formation.

Table 1. Characteristics of black pepper shoots submitted to *ex vitro* rooting

Cultivar	Stem Length (mm)	Number of Leaves	Number of buds
Apra	11.96 a	2.00 a	2.43 a
Bragantina	11.40 a	2.33 a	2.28 a
Kuthiravally	11.76 a	2.17 a	2.40 a
CV %	12.80	17.11	15.83

Shoot responses regarding rooting and survival rates were similar in relation to NAA doses. The concentration of 80.56 µmol/L provided 100% rooting and survival in the acclimatization of ‘Bragantina’ and ‘Kuthiravally’ cultivars (Figure 1). When cultivated *in vitro* and submitted to high auxin doses, shoots can impair plant survival due to toxicity. This is because phytoregulators act at extremely low concentrations, in the order of milligrams or micromolar, which can inhibit, modify or promote plant growth.

Figure 1. Survival rate and rooting for the three black pepper cultivars.



Thus, it was observed that the auxin concentration (NAA) influenced the rooting and growth of black pepper cultivars during acclimatization. Studies have shown that this species requires exogenous application of auxins in the rooting process, whether in *in vitro* Ravindran et al. (2016), or *ex vitro* cultivation. According to Secundino et al. (2014) and Ambrozim et al. (2017), concentration may vary according to the mode of application, usually in the liquid form, and the tendency is for the use of lower concentrations.

The action of auxins occurs initially at cell level, in cell expansion and elongation, improving cell division, mainly in rooting (Ford et al., 2001; Zeny and Trojan, 2016). The stress caused on shoots through explant injury and exogenous application of auxin alters the endogenous hormonal balance, and creates a new balance (Guo et al., 2009), which may be beneficial for root induction along with the action of NAA at cell level.

The responses of cultivars in relation to NAA doses, according to analysis of variances for variables NR, LR, NL and LSDM, revealed the cultivar *versus* NAA doses interaction, indicating that the effects of NAA concentrations depend on genotype (cultivar), with specific dose responses in relation to genotype, and in order to understand this specificity, it is necessary to analyze the results of this interaction. On the other hand, RDM does not depend on NAA doses to which shoots were immersed, and responses indicated differences according to cultivar, indicating that each genotype has its competence in rhizogenesis (Table 2).

Cultivars have different induction responses to the number of roots in each applied NAA dose. Furthermore, the behavior of ‘Bragantina’ and ‘Kuthiravally’ cultivars in relation to NAA doses was specific for each dose, that is, NR varied according to the applied dose; in the ‘Apra’ cultivar, regardless of NAA dose, roots were induced in a similar way, without statistical difference, considering the evaluation of doses within each cultivar. Similar fact occurred for root length, where each dose promoted different growth among cultivars, as well as cultivars in relation to all NAA doses, except for the 'Apra' cultivar, in which the dose effect for root length was similar, unlike the result observed for 'Bragantina' and 'Kuthiravally' cultivars, which had different reactions in relation to the applied doses.

Table 2. Summary of analysis of variance of variables rooting and shoot development during acclimatization of three black pepper cultivars after six weeks of cultivation.

Source of Variation	Degrees of Freedom	Mean Square									
		RN		RL		LN		RDM		LSDM	
Block	4	0.642	ns	8.64	ns	2.85	*	0.15	ns	42.8	ns
Cultivar (C)	2	32.55	**	2384.14	*	1.87	ns	29.18	*	1049.15	*
Dose (D)	3	5.8	**	77.64	*	1.64	ns	0.62	ns	656.52	*
C * D interaction	6	2.683	**	102.95	*	4.31	*	0.54	ns	417.74	*
Residue	44	0.505		19.36		0.9		0.36		38.6	
Total	59	2.09		110.25		1.45				8444.1	
CV (%)		**22.93**		**25.19**		**31.01**		**31.04**		**15.54**	

NR: number of roots; RL: root length. NL: number of leaves; RDM: root dry matter and LSDM: shoot dry matter.

The effect of each dose was also reflected in the number of leaves for the different cultivars, except for the dose of 53.70 µmol/L, in which all cultivars had similar behavior. On the other hand, 'Apra' and 'Bragantina' cultivars presented similar number of leaves, indicating that NAA doses do not significantly affect this characteristic. On the other hand, 'Kuthiravally' cultivar responded to NAA doses with different number of leaves (Table 3). The results revealed that NAA doses have different effect on the development of black pepper cultivars regarding variables number of roots, root length, number of leaves and shoot dry matter.

Table 3. Results of the analysis of variance of variable rooting in the acclimatization phase of three black pepper cultivars submitted to application of four NAA doses after six weeks of cultivation.

Source of Variation	Degrees of Freedom	Mean Square							
		RN		RL		RDM		LSDM	
C: Dose 26.85 µmol/L	2	6.067	*	190.18	*	4.87	*	41.9	ns
C: Dose 53.70 µmol/L	2	12.2	*	874.57	*	0.87	ns	173.6	*
C: Dose 80.56 µmol/L	2	7.47	*	515.09	*	3.8	*	634.6	*
C: Dose 134.26 µmol/L	2	14.87	*	1113	*	5.82	*	1453	*
D: 'Apra'	3	0.27	ns	3.25	ns	1.73	ns	339.2	*
D: 'Bragantina'	3	8.72	*	167.13	*	1.73	ns	33.58	ns
D: 'Kuthiravally'	3	2.18	*	113.17	*	6.8	*	119.2	*
Residue	44	0.5		19.35		0.9		38.6	

C: cultivar and D: doses

The means comparison tests showed that for variable NR at NAA dose of 26.85 µmol/L, 'Bragantina' and 'Kuthiravally' cultivars promoted the highest means in relation to 'Apra' cultivar; while at NAA doses of 53.70 and 134.26 µmol/L, 'Kuthiravally' cultivar behaved differently from the other cultivars; and for NAA dose of 80.56 µmol/L, 'Apra' and 'Kuthiravally' cultivars had similar responses that differed from 'Bragantina' cultivar (Figure 2A). It should be highlighted that there was an effect of NAA dose on 'Bragantina' and 'Kuthiravally' cultivars and 'Bragantina' cultivar, at NAA dose 26.85 µmol/L stood out from the other cultivars; and in 'Kuthiravally' cultivar, NAA doses of 26.85 and 53.70 µmol/L promoted significant effects (Figure 2B).

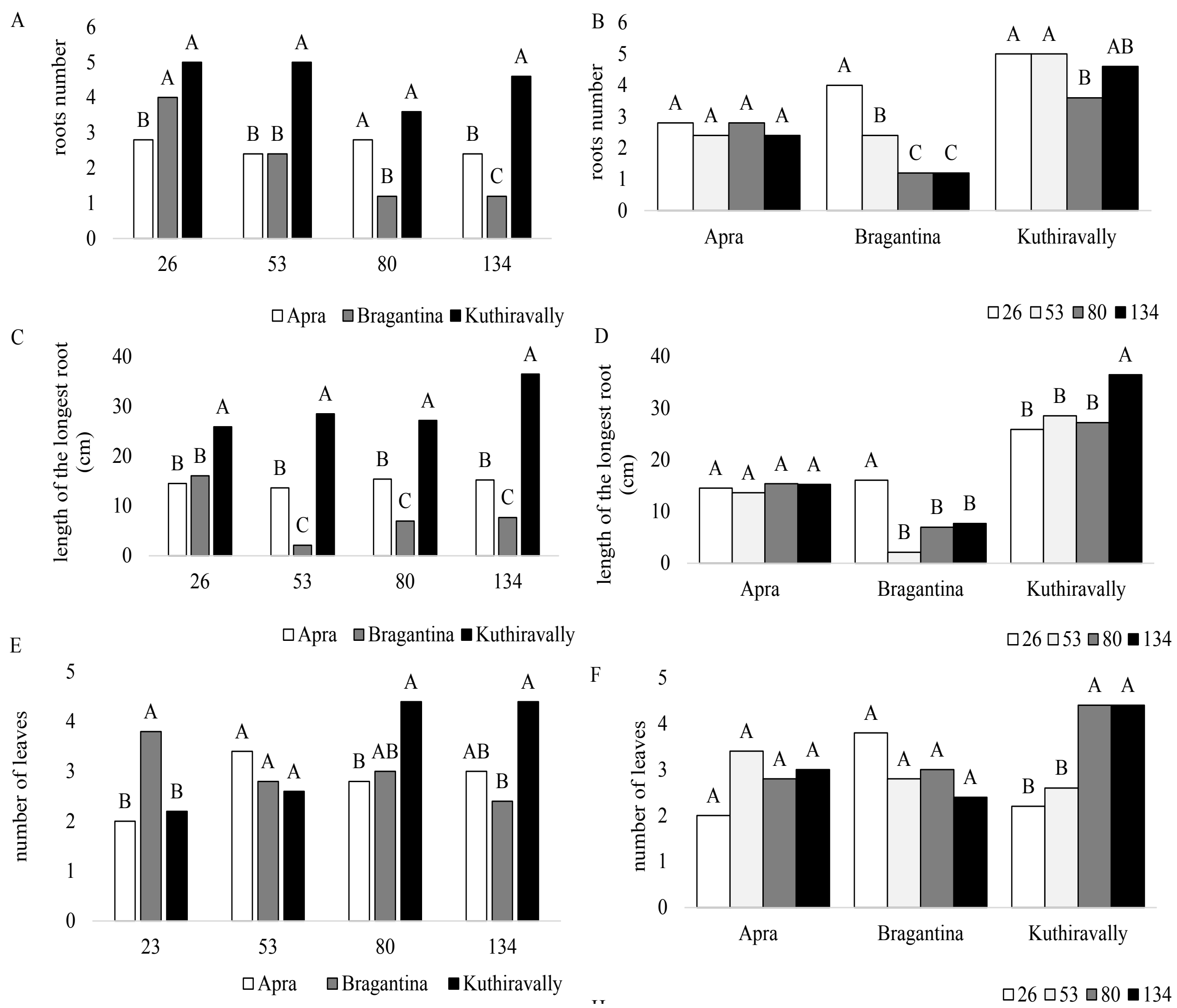
In addition to promoting root induction, auxin affects growth. In this case, for root length at all doses, 'Kuthiravally' cultivar had higher averages, differing from the other cultivars (Figure 2C). For the root length of each cultivar, the effect of NAA doses differentiated root length in 'Bragantina' and 'Kuthiravally' cultivars, when applying doses of 26.85 µmol/L in 'Bragantina' shoots and 134.26 µmol/L in 'Kuthiravally' ', which promoted greater root growth (Figure 2D).

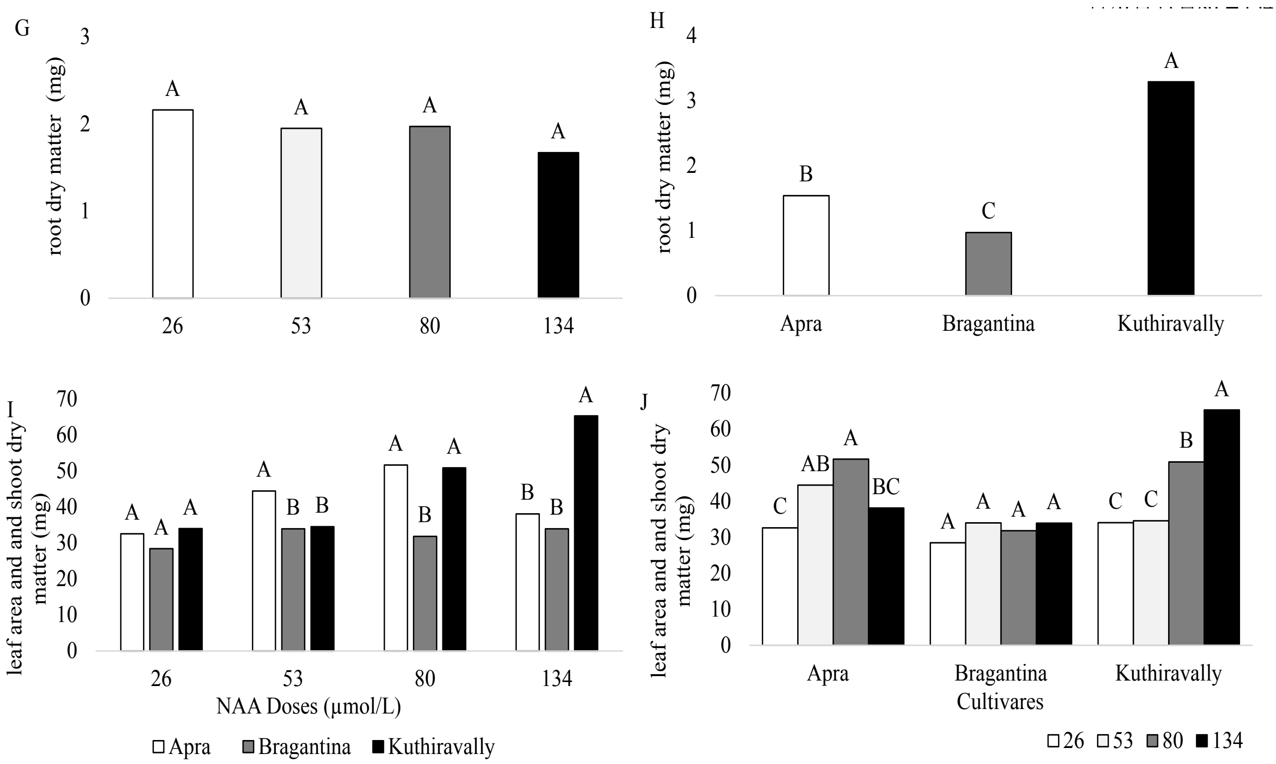
As for number of leaves, it was observed that NAA doses have little effect on this variable and that this characteristic is more related to genotype (cultivar). Cultivars only had similar behavior regarding leaf emission at NAA dose of 53.70 µmol/L; while at dose of 26.85 µmol/L, 'Bragantina' emitted more leaves; at dose of 80.56 µmol/L, 'Kuthiravally' emitted more leaves than 'Apra' cultivar, but similar to 'Bragantina'; and at dose of 134.26 µmol/L, 'Kuthiravally' again stood out; however, differing only from 'Bragantina' cultivar (Figure 2E). Cultivars in which the average number of leaves were similar in most doses, with the exception of 'Kuthiravally' cultivar, stood out at NAA doses of 80.56 and 134.26 µmol/L, with higher leaf emission (Figure 2F).

Considering variable RDM, 'Kuthiravally' cultivar differed from the others, exhibiting the highest mean root dry matter values (Figure 2G). In relation to NAA doses, cultivars showed similar behavior (Figure 2H).

Regarding variable LSDM, at dose of 26.85 µmol/L, the responses of cultivars were similar, but at NAA dose of 53.70 µmol/L 'Apra' cultivar differed from the other cultivars, and for doses of 80, 56 and 134.26 µmol/L, 'Kuthiravally' cultivar differed from the others, showing the highest mean values; however, at dose of 80.56 µmol/L, this cultivar was similar to 'Apra' cultivar (Figure 2I). In each cultivar, influence of NAA doses was observed. At NAA dose of 80.56 µmol/L 'Apra' cultivar exhibited the highest LSDM values, but it did not differ only from NAA dose of 53.70 µmol/L, and regardless of the dose applied, 'Bragantina' responses were similar and for the 'Kuthiravally' cultivar, the highest dose stood out in relation to this variable (Figure 2J).

Figure 2. Development of three *P. nigrum* cultivars in the rooting and acclimatization phase of shoots submitted to different NAA doses after six weeks of cultivation.





Ravindran et al. (2016) and Secundino et al. (2014) demonstrated that auxins were necessary to induce rhizogenesis in species of the genus *Piper*, which act on cell elongation and expansion as signals for the formation of root primordia, mainly in micropropagation.

According to Souza and Pereira (2007), when auxins are applied at the cutting base, they are translocated to different organs of the plant up to leaves, where the activation of the indoleacetic acid (IAA) occurs, which will later be transported to the root initiation region.

The application of exogenous auxin (NAA) promoted the induction and development of the root system of the three black pepper cultivars, with 'Kuthiravally cultivar being more sensitive to the action of NAA. Serrano et al. (2012) showed the difference in response of genotypes in the production of black pepper seedlings, via cuttings, through shoot and root dry matter accumulation and number of leaves.

Genotype response has been important for plant tissue culture, as each genotype has an intrinsic physiological effect (Shahzad, 2017). With these results, it was observed that *ex vitro* rooting, in the acclimatization phase of black pepper, was influenced by genotype, as observed in the conventional propagation method through the rooting of cuttings.

Freire et al. (2017) verified that by treating the base of black pepper cuttings with auxins, it was possible to accelerate their development and increase the number and quality of roots formed in the cuttings, since the quality of the root system in *Piper nigrum* cuttings is highly associated with adventitious root induction. In this study, auxin (NAA) promoted rooting and the number of root primordia and root growth, characteristics related to the quality of the root system of black pepper seedlings, whether from *in vitro* cultivation or from the rooting of cuttings by the conventional method.

According to Souza and Pereira (2007), the presence of leaves in cuttings is important for rooting, as they are essential for the production of nitrogen compounds, nutrients or auxins essential for root formation. Thus, it was observed that, in addition to microcuttings having adequate size, they must have sufficient number of leaves to help in the rhizogenesis process.

A characteristic of the 'Kuthiravally' cultivar is that it has wide and long leaves (Lemos et al., 2011). Regarding the number of leaves 'Kuthiravally' cultivar obtained higher values, both in the different NAA concentrations applied to shoots, and when compared to the other cultivars. The number of leaves influences the rooting rate due to the synthesis of carbohydrates and other substances required for rooting, thus, plants with greater number of leaves are photosynthetically more efficient, as they present greater leaf area (Suthar and Puroh, 2012).

Auxins are linked to RNA synthesis and cell division, promoting the initiation of root primordia, and are necessary for the development of new root tissues and, consequently, stimulate plant growth (Zeny and Trojan, 2016), therefore, they play an important role in root induction and seedling formation.

Shoot rooting at the acclimatization phase of the three cultivars after immersion in NAA solution is an indicated procedure for black pepper cultivation. There is a difference in the responses of cultivars regarding the applied NAA doses, and the 'Kuthiravally' cultivar is more sensitive to the action of this auxin for rhizogenesis, suggesting the use of lower concentrations to reduce production costs.

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