Universidade Federal Rural do Semi-Árido
Pró-Reitoria de Pesquisa e Pós-Graduação
https://periodicos.ufersa.edu.br/index.php/caatinga
ISSN 1983-2125 (online)

http://dx.doi.org/10.1590/1983-21252024v3712180rc

Scientific Article

Combined effect of cytokinins on the *in vitro* propagation of three strawberry cultivars

Efeito combinado de citocininas na propagação *in vitro* de três cultivares de morango

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ABSTRACT - In the *in vitro* propagation of strawberry plants, techniques have been applied to obtain disease-free plants with high multiplication rates. To improve the efficacy of micropropagation protocols, it is necessary to determine the effect of growth regulators, mainly cytokinins; however, there is no information regarding the combined use of trans zeatin (Zt) and other cytokinins for shoot induction. The objective of this work was to evaluate the effect of Zt combined with 6-benzylaminopurine (BAP), 6-furfurylaminopurine (KIN) or 2-isopentenyladenine (2iP) on the *in vitro* propagation of three strawberry cultivars. Combinations of cytokinins, Zt and BAP, Zt and KIN, and Zt and 2iP were proposed to evaluate shoot induction. For the acclimatization of the seedlings, two types of substrates were used (Pro-Mix, Pro-Mix plus humus 2:1). The use of Zt and KIN increased the average number of shoots per explant, and the greatest number of leaves and roots was obtained when Zt and 2iP or Zt and BAP were used. The Pro-Mix and Pro-Mix plus humus substrates did not significantly affect the number of leaves or roots, or the SPAD. These results indicate that the use of the combination of Zt and KIN allowed us to obtain the greatest number of shoots per explant that did not produce calli and promoted the development of the root system; likewise, the Pro-Mix or Pro-Mix plus humus substrate were adequate for acclimatization, allowing the growth and development of strawberry plants.

Keywords: *Fragaria*. Plant growth regulator. Micropropagation. SPAD index.

RESUMO - No cultivo *in vitro* do morango são aplicadas técnicas de micropropagação para obtenção de plantas livres de doenças e com altas taxas de multiplicação. Para melhorar a eficácia dos protocolos de micropropagação é necessário conhecer o efeito dos reguladores de crescimento, principalmente as citocininas, porém não há informações a respeito do uso combinado da transzeatina (Zt) com outras citocininas para indução de brotações. O objetivo deste trabalho foi avaliar o efeito combinado de Zt com 6-benzilaminopurina (BAP), 6-furfurilaminopurina (KIN) ou 2-isopenteniladenina (2iP) na propagação *in vitro* de três cultivares de morangueiro. Combinações de citocininas, Zt e BAP, Zt e KIN, Zt e 2iP foram propostas para avaliar a indução de brotos. Na aclimatação das mudas foram utilizados dois tipos de substratos (Pro-Mix, Pro-Mix e húmus 2:1). Observou-se que o uso de Zt e KIN induziu maior número de brotos por explante, o maior número de folhas e raízes foi obtido quando foram utilizados Zt e 2iP ou Zt e BAP. O substrato Pro-Mix ou Pro-Mix e humus não apresentou diferenças significativas para o número de folhas e raízes, e elevados valores de índice SPAD. Os resultados indicam que o uso da combinação de Zt e KIN permitiu obter o maior número de brotos por explante que não produziram calos e promoveram o desenvolvimento do sistema radicular, da mesma forma, o substrato Pro-Mix ou Pro-Mix e humus foi adequado para aclimatação permitindo crescimento e desenvolvimento de mudas de morango.

Palavras-chave: *Fragaria*. Regulador de crescimento. Micropropagação. Índice SPAD.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.

Received for publication in: September 8, 2023.
Accepted in: April 2, 2024.

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INTRODUCTION

The strawberry (*Fragaria* × *ananassa* Duch.) is a hybrid obtained in Europe more than 300 years ago from a cross between *Fragaria virginiana* and *Fragaria chiloensis*, (JHAJHRA et al., 2018). It is an herbaceous plant belonging to the Rosaceae family and is usually propagated vegetatively from a runner; however, this method is limited because it is laborious, time-consuming and involves the transmission of strawberry viruses (SEHRAWAT et al., 2016; QUIROZ et al., 2017; TORRICO et al., 2018). Currently, *in vitro* propagation is being used, which can be used throughout the year, and the culture of nodal segments *in vitro* has become the most widely used technique in strawberry micropropagation (KARHU; HAKALA, 2002).

In vitro propagation results in the greatest number of uniform seedlings, greater survival in the field, and an increase of up to 24% in yield compared to conventionally propagated plants (KIKAS; LIBEK; VASAR, 2006). However, this type of unconventional propagation requires the use of growth regulators such as auxins and cytokinins to induce organogenesis, which must be evaluated to optimize their use (HADDADI et al., 2010). There are reports on the exclusive use of cytokinins alone or in combination for the induction of adventitious shoots in the genus *Fragaria*. For example, for the shoots of *F. chiloense* Chilean strawberry plants placed in MS basal media supplemented with 0.5 mg L^{-1} BAP, it was possible to induce 6 adventitious shoots (QUIROZ et al., 2017) In *F. viridis* the use of MS basal media supplemented with 0.5 mg L^{-1} thidiazuron (TDZ) and

0.1 mg L^{-1} BAP allowed the acquisition of 14 adventitious shoots (GHASEMI et al., 2015). In *Fragaria* x *ananassa* cv. Aromas, growth in MS media supplemented with 1.0 mg L^{-1} of zeatin induced 4.2 shoots (NERI et al., 2022). In the cv. Chandler, the use of MS basal media supplemented with 1.5 mg L^{-1} TDZ induced 5.7 adventitious shoots (IRSHAD et al., 2023). In the cv. Camarosa the use of MS basal media supplemented with 0.5 mg L^{-1} TDZ and 1.0 mg L^{-1} BAP induced 14 adventitious shoots (HADDADI et al., 2010). In the Sweet Charlie and Winter Dawn cultivars, MS culture plus 5.0 mg L^{-1} BAP and 0.1 mg L^{-1} KIN induced 5 adventitious shoots (DHUKATE et al., 2021). The use of cytokinins combined with auxins in cv. Ofra in MS/2 media supplemented with 2.5 mg L^{-1} KIN and 0.1 mg L^{-1} indoleacetic acid (IAA) induced 9.2 adventitious shoots (SEHRAWAT et al., 2016), and with cytokinins combined with gibberellin, as is the case for the Marquez cultivar, it was possible to induce 4 to 6 adventitious shoots using MS basal media plus 0.1 mg L^{-1} BAP and 0.4 mg L^{-1} AG_3 (MOHAMED et al. al., 2016). The use of combined cytokinins has had an important effect on the induction of shoot growth in strawberry plants, making it necessary to evaluate the effect of the combined use of Zt with other cytokinins. In this context, the present work evaluated the combined effect of Zt with BAP, KIN or 2iP on the *in vitro* propagation of three strawberry cultivars.

MATERIAL AND METHODS

Plant material

In the present investigation, strawberry seedlings of the Aromas, Camino Real and Sabrina cultivars established *in vitro* from meristems in 2021 in the Biology Laboratory, Universidad Nacional Toribio Rodríguez de Mendoza, Peru, were used.

Induction of adventitious shoots in strawberry cultivars

The basal shoots of the strawberry cultivars were cultivated for five weeks in multiplication media supplemented with 30 g L^{-1} sucrose, 1.0 g L^{-1} myo-inositol, 300 mg L^{-1} ascorbic acid, 0.5 mg L^{-1} naphthaleneacetic acid (ANA) and 5.0 mg L^{-1} BAP, and 1.5 g L^{-1} phytagel (Sigma). The pH of the medium was adjusted to 5.0 using NaOH and 1 N HCl solutions before autoclaving at 121 ºC for 15 minutes. The induced shoots were transferred to MS media supplemented with 30 g L^{-1} sucrose and 100 mg L^{-1} myo-inositol (pH adjusted to 5.8) without growth regulators for five weeks. The photoperiod conditions were 16/8 hours of light/dark provided by cool white fluorescent tubes for illumination with an intensity of 75 µmol $m^{-2}s^{-1}$.

Well-formed basal shoots with two to three true leaves were used for the induction of adventitious shoots in WPM media with a composition similar to that of the multiplication media, except for the combinations of Zt cytokinins (1.0 mg L^{-1}) with BAP, KIN or 2iP (1.0 mg L^{-1}). After six weeks of *in vitro* culture, the number of shoots, leaves and roots per explant were collected.

Seedling acclimatization in strawberry cultivars

The induced basal shoots of the three strawberry cultivars with two to three true leaves were cultivated in MS media without growth regulators, and 12 seedlings were cultured in each magenta vessel. The shoots from five weeks of culture were used to produce induced leaves and roots.

The rooted plants were transferred into trays with 40 alveoli filled with Pro-Mix or Pro-Mix plus humus substrate (2:1 ratio), placed in an environment with 90% relative humidity, and a photoperiod of 16/8 hours light/dark provided by cool white fluorescent tubes for illumination with an intensity of 75 µmol $m^{-2}s^{-1}$, and irrigated at field capacity with hydroponic solutions. After six weeks of acclimatizing the strawberry plants, data on the number of leaves, and roots, and the SPAD index were collected.

Statistical analysis

To evaluate the effects of shoot induction and seedling acclimatization on the three strawberry cultivars, both experiments were conducted using a factorial experiment with a completely randomized design. In the induction of adventitious shoots, the response variables were evaluated through a 3A x 3B bifactorial experiment (Factor A: strawberry cultivars, Factor B: combinations of Zt with BAP, KIN, or 2iP), with five repetitions, each consisting of five explants. During seedling acclimatization, the response variables were evaluated through a 3A x 2B bifactorial experiment (Factor A: strawberry cultivars, Factor B: type of substrate), with twenty repetitions per treatment. For both experiments, two-factor ANOVA was used to analyze the combined effects of the factors and the means were calculated using the Tukey test at $P \leq 0.05$. The data on the number of shoots and number of roots (induced adventitious shoot and seedling acclimatization, respectively) were transformed using $(x + 0.5)^{1/2}$. The bifactorial MANOVA was performed with the variables established in both experiments of the present study. Significant differences were considered at $P \leq 0.05$ and $P \leq 0.01$. The relationship between the variables was explored using principal component analysis, the results of which were visualized in a biplot built between the first two principal components (PC1 and PC2). The statistical analysis of the data was performed with R software version 4.1.0.

RESULTS AND DISCUSSION

Induction of adventitious shoots in strawberry cultivars

The two-way MANOVA of the induction of adventitious shoots in strawberry revealed a significant multivariate main effect for cultivar, cytokinin, and the double interaction cultivar x cytokinin (p≤0.001) (Table 1). Based on these test results, the univariate main effects were independently examined for each variable (Table 2).

Table 1. Two-way MANOVA of the main effects of strawberry cultivar, cytokinin, and the combined effects on the response variables evaluated (number of shoots, roots, and leaves).

Variable	Wilks' Lambda	F	*p*
Cultivar	0.34	7.98	<0.001
Cytokinin	0.16	16.66	<0.001
Cultivar x Cytokinin	0.39	3.26	<0.001

Table 2. Two-way interaction ANOVAs for the induction of adventitious shoots in three strawberry cultivars. The factors included cultivar (Cu) Aromas, Sabrina, and Camino Real; and cytokinin (Cy) Zt combined with BAP, KIN, or 2iP. The response variables were measured after six weeks of *in vitro* culture.

Response Variable	Factor	d.f.	F	*p*
Number shoots	Cultivar (Cu)	2	0.78	0.47
	Cytokinin (Cy)	2	47.24	<0.001
	Cu x Cy	4	1.83	0.14
	Residuals (R)	36		
Number roots	Cu	2	23.80	<0.001
	Cy	2	42.14	<0.001
	Cu x Cy	4	6.30	<0.001
	R	36		
Number leaves	Cu	2	22.69	<0.001
	Cy	2	29.50	<0.001
	Cu x Cy	4	2.14	0.09
	R	36		

Figure 1 shows the comparison of the means of the variables evaluated in the induction of shoot growth in the three strawberry cultivars. Regarding the number of shoots per seedling, the use of both Zt and KIN (1.0 mg L^{-1}) induced a greater number of shoots per explant in the strawberry cultivar, Sabrina (21 shoots), Aromas (18 shoots), and Camino Real (15 shoots); however, the number of shoots, there were no significant differences among the strawberry cultivars evaluated. The combined use of cytokinins has been shown to be effective in inducing a greater number of shoots than TDZ alone (BHATT; DHAR, 2000). A similar effect has been recorded in *Chlorophytum borivilianum* with the combined use of the cytokinins BAP and KIN, which resulted in approximately double the number of shoots (ASHRAF et al., 2014). In strawberry plants, the combined effects of cytokinins on the Camarosa cultivar include the use 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} for 15 shoots per explant (HADDADI et al., 2010), the use 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} KIN for 16.6 shoots per explant (ARA et al., 2012), the use of the same concentrations and combinations of cytokinins in the Sweet Charlie cultivar for 9.3 shoots per explant (HARUGADE; TABE; CHAPHALKAR, 2014). In this study, the combined use of the cytokinins Zt and KIN had a synergistic effect, resulting in four times more shoots (18.6 shoots) in the Aromas cultivar than that reported by Neri et al. (2022), who obtained 4.2 shoots from each explant using only zeatin.

With the combination of the cytokinins Zt and KIN (both 1.0 mg L^{-1}) the explants did not produce calluses in the three strawberry cultivars studied, allowing good quality shoots to be obtained. In contrast, when using TDZ or zeatin at concentrations above 0.25 mg L^{-1} in the Bounty cultivar, DEBNATH (2006) increased the number of shoots but also increased the size of the basal callus; this response was greater when using TDZ than when using zeatin. In *Bauhinia vahlii* explants, the use of the ANA auxin in combination with TDZ produced excessive callus (BHATT; DHAR, 2000). The production of calli in explants does not allow adequate application in the regeneration of seedlings from callus tissue because it frequently increases somaclonal variation, which is undesirable in the production of planting materials. The lack of callus formation in the present study indicates that the combination of Zt and KIN is suitable for the mass production of strawberry plants.

The number of roots of the induced shoots differed among the cultivars in this study. In the Aromas cultivar, the greatest number of roots was induced when Zt and 2iP (both 1.0 mg L^{-1}) or Zt and BAP (both 1.0 mg L^{-1}) were used; in the Sabrina cultivar, the greatest number of roots was obtained when Zt and BAP or Zt and 2iP were used (both 1.0 mg L^{-1}), whereas in the Camino Real cultivar, the number of roots did not significantly differ among the combinations of cytokines used. Although all explants in which adventitious shoots were induced formed roots when transferred to MS media without growth regulators, the combinations of Zt and BAP (both 1.0 mg L^{-1}) or Zt and 2iP (both 1.0 mg L^{-1}), promoted better development of the root system, not requiring the seedlings to be transferred to root induction media.

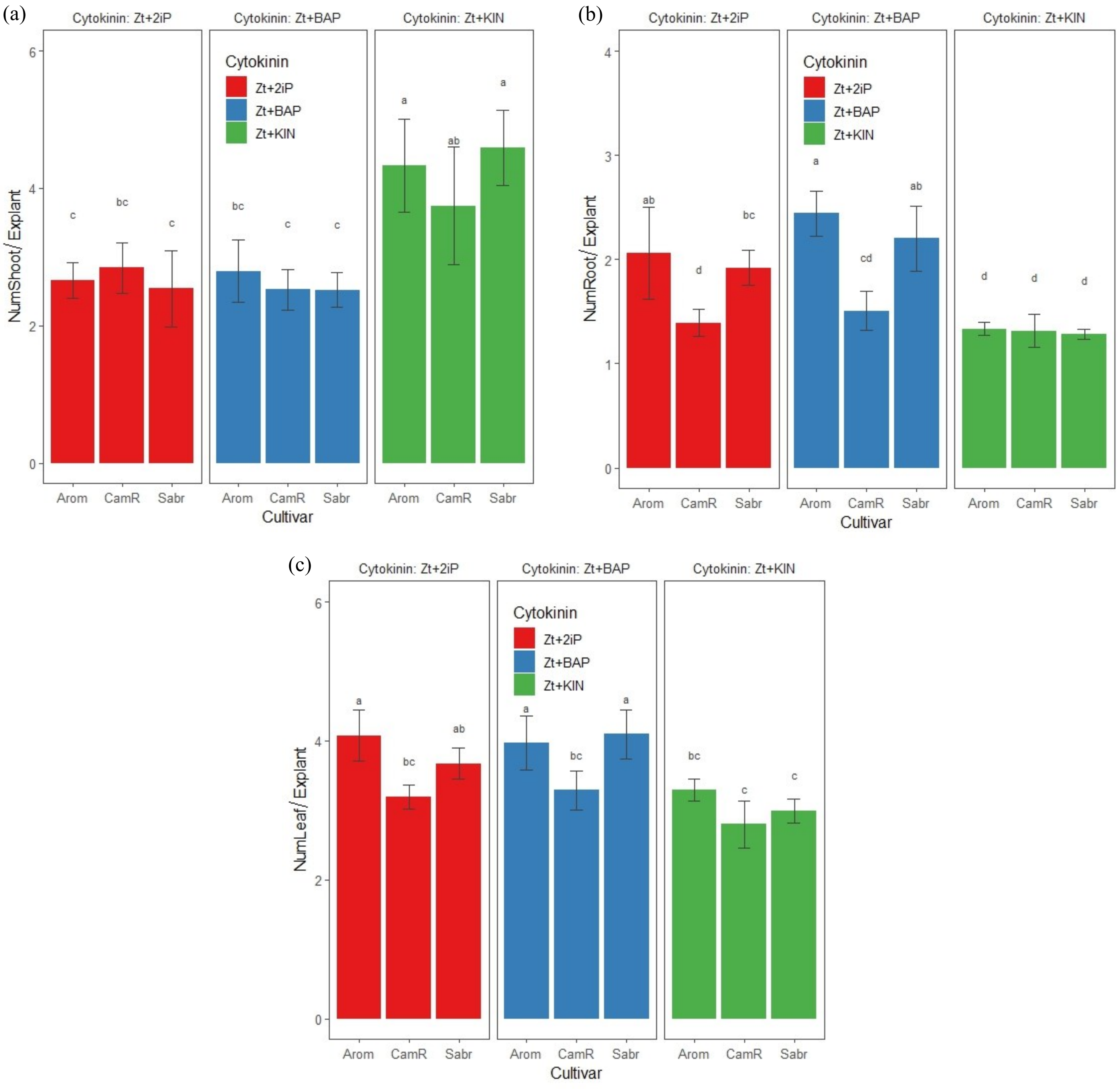
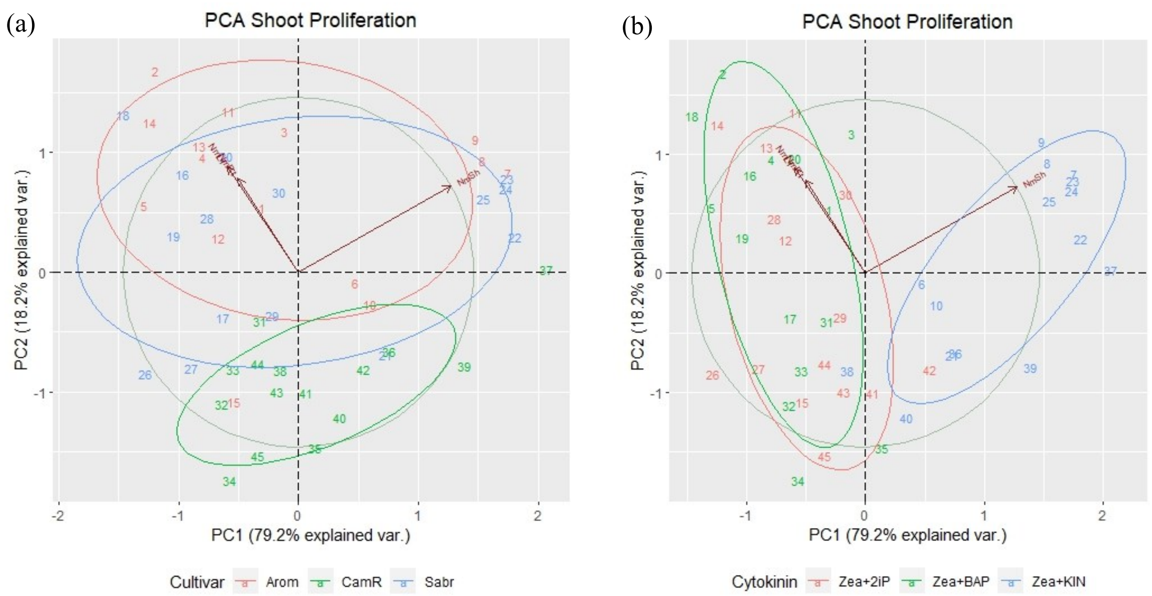


Figure 1. Shoot induction in strawberry cultivars. a) Number of shoots, b) Number of roots, c) Number of leaves. The data are presented as the means ± standard deviations, and different letters indicate significant differences in the parameters for p≤0.05 according to the Tukey test.

Zeatin has not been used for the rooting of strawberry shoots, and the protocols established for this species use basal media supplemented with cytokinins for shoot multiplication; subsequently, shoot rooting is carried out *ex vitro* or *in vitro* (DEBNATH, 2006). Similarly, positive effects have been described in the *in vitro* propagation of the strawberry Aromas cultivar, which root formation is regulated in a multiplication medium supplemented with zeatin (1.0 mg L^{-1}) to promote the greatest development of the root system (NERI et al., 2022). Additionally, in the Bounty cultivar, DEBNATH (2006) used only 0.5 and 1.0 mg L^{-1} zeatin and obtained the same results. In this study, Zt and KIN were added to WPM basal media for shoot multiplication, and the plants were subsequently transferred to regulator-free MS basal media. The greatest number of uniform and rooted seedlings was obtained after 6 weeks of *in vitro* culture, and the number of uniform and rooted seedlings increased during the acclimatization stage. This approach is important for optimizing production costs and reducing the time needed to obtain plant material from *in vitro* sources.

The shoot apical meristems are highly organized tissues that contain pluripotent stem cells with the capacity to divide into different functional zones such as the central zone, peripheral zone, and rib zone. Leaf primordia begin in the peripheral zone, where cells undergo differentiation (WU et al., 2021). In different studies, cytokinins have been shown to be regulators of leaf initiation; in this way, in tomato, the use of 0.2 mg L^{-1} of zeatin in the apical meristems of shoots results in the formation of leaf primordia (YOSHIDA; MANDEL; KUHLEMEIER, 2011). Considering that cytokinins play an important role in the formation of leaf primordia from the apical meristems of the shoot, it can be understood that the use of the cytokinins zeatin or TDZ influences the greatest number of leaves per explant in *in vitro* propagation for example, in *Vaccinium arctodtaphylos* the use of zeatin or TDZ (both 2 mg L^{-1}) plus 0.1 mg L^{-1} indole butyric acid induced the greatest number of leaves (16 and 11 leaves, respectively) compared with when they were not used (CÜCE; BEKTAŞ; SÖKMEN, 2013). In the Aromas strawberry cultivar, the use of 1 mg L^{-1} of zeatin doubled the number of leaves compared to the control (17 leaves compared to 9 leaves in the control) (NERI et al., 2022). In the present investigation, the number of leaves per explant differed among the cultivars studied. In the Aromas cultivar, the greatest number of leaves was induced when Zt and 2iP (both 1.0 mg L^{-1}) or Zt and BAP (both 1.0 mg L^{-1}) were used, and in the Sabrina cultivar, the greatest number of leaves was obtained when Zt and BAP or Zt and 2iP were used (both 1.0 mg L^{-1}). Meanwhile, in the Camino Real cultivar, the number of leaves did not significantly differ among the combinations of cytokines used. The number of leaves induced in the present work was four when Zt and BAP or Zt and 2iP were used in the Aromas and Sabrina cultivars, a few leaves below those recorded by Neri et al. (2022) in the Aromas cultivar. The lower number of leaves recorded in the present investigation can probably be explained by the fact that the combinations of cytokinins had a greater influence on the number of shoots than on the number of leaves; these variables were highly correlated, but inversely. As the number of shoots increased, the number of leaves decreased (Figure 2).

The shoot proliferation response of strawberry cultivars in which Zt was combined with BAP, KIN or 2iP was evaluated through principal component (PC) analysis, and PC1 and PC2 explained 97.4% of the total variation in the data. PCA revealed different responses in the proliferation of adventitious shoots. Therefore, the proliferation of the shoots of the cultivars Aromas and Sabrina was similar to that of the cultivar Camino Real (based on positive scores of PC1). The graph of the association of Zt combined with KIN revealed a better response in terms of shoot proliferation than when Zt was combined with BAP or 2iP (Figures 2a and 2b). These results demonstrated that the Aromas and Sabrina cultivars exhibited notable physiological changes in the induction of shoot growth by the combined action of Zt and KIN. Therefore, cytokinins play an important role in controlling the balance of the root: shoot ratio (SILVA-NAVAS et al., 2019), and the synergistic effects of the combination of Zt and KIN may occur because zeatin was found to be an effective cytokinin for the induction of shoot growth, probably because of the presence of zeatin reductase, converts zeatin to dihydroxyzeatin, which is not attacked by cytokinin oxidase/ dehydrogenase, maintaining its biological activity in organogenesis in plants (NIKOLIĆ et al., 2006). On the other hand, it is likely that the synergistic effect of Zt and KIN on the greatest shoot induction by strawberry explants may be because this combination of cytokinins is related to the inhibitory effect of KIN on ethylene production. This synergistic effect has been reported previously when a combination of BAP and KIN cytokinins was used; since BAP triggers the release of a large amount of ethylene in *in vitro* culture, such as in *Lagenaria siceraria* (SAHA; MORI, H.; HATTORI, 2007) and *Chlorophytum borivilianum* (ASHRAF et al., 2014), and this is inhibited by KIN. Both hypotheses need to be investigated in strawberry.



Arom (Aromas), CamR (Camino Real), and Sabr (Sabrina)
NmSh (number of shoots), NmRt (number of roots), NmLf (number of leaves)

Figure 2. Main components of strawberry shoot induction. a) Graph demonstrating the degree of association and divergence in strawberry cultivars. b) Graph demonstrating the degree of association and divergence in the use of Zt in combination with BAP, KIN or 2iP.

***Ex vitro* acclimatization of strawberry cultivars**

The two-factor MANOVA of the *ex vitro* acclimatization of strawberry seedlings revealed a significant multivariate main effect for cultivar, substrate, and the double interaction cultivar x substrate ($p \leq 0.01$) (Table 3). Based on these test results, univariate main effects were examined for each variable independently (Table 4).

Table 3. Two-way MANOVA of the main effects of strawberry cultivar, substrate and their combination on the response variables evaluated (number of leaves, number of roots, and SPAD index).

Variable	Wilks' Lambda	F	*p*
Cultivar	0.59	11.13	<0.001
Substrate	0.82	8.37	<0.001
Cultivar x substrate	0.76	5.37	<0.001

Table 4. Two-way interaction ANOVAs for the acclimatization of strawberry plants. The factors included cultivar (Cu) Aromas, Sabrina, and Camino Real; substrate (Su) Pro-Mix or Pro-Mix plus humus. The response variables were measured after six weeks of *in vitro* culture.

Response variable	Factor	d.f.	F	*p*
Number leaves	Cultivar (Cu)	2	4.14	0.018
	Substrate (Su)	1	16.24	<0.001
	Cu x Su	2	12.60	<0.001
	Residuals (R)	114		
Number roots	Cu	2	14.86	<0.001
	Su	1	1.61	0.21
	Cu x Su	2	1.78	0.17
	R	114		
SPAD index	Cu	2	17.71	<0.001
	Su	1	2.11	0.15
	Cu x Su	2	3.90	0.023
	R	114		

Figure 3 shows the comparison of the means of the variables evaluated in the seedling acclimatization stage for the three strawberry cultivars, revealing that the plants sown in the Pro-Mix substrate presented better growth and development, as determined by the greater number of leaves. The Aromas and Sabrina cultivars did not show significant differences when using the two types of substrates, while for the Camino Real cultivar, the Pro-Mix substrate had the greatest number of leaves compared with the Pro-Mix plus humus substrates. However, the number of roots and the SPAD index, were similar among the strawberry cultivars evaluated.

The response of strawberry cultivar seedlings subjected to Pro-Mix or Pro-Mix and plus humus substrate to acclimatization was evaluated through principal component analysis (PC), and PC1 and PC2 explained 99.5% of the total variation in the data. PCA revealed differences in the responses of the strawberry seedlings to acclimatization in the two types of substrates. Therefore, the Aromas and Sabrina cultivars showed similar effects on the acclimation of the seedlings in comparison with the Camino Real cultivar. The graph of the types of substrates did not show differences in the acclimatization of strawberry seedlings (Figures 4a and 4b). However, these results show that the Aromas and Sabrina cultivars exhibited satisfactory adaptation responses in the acclimatization process of strawberry plants.

In the acclimatization stage, the seedlings must present a well-developed root system (DEWIR et al., 2015); in such a way that it allows adequate assimilation of water and nutrients. In the present study, seedlings with a good root system were used, allowing 100% survival of the acclimatized plants to be achieved. The Pro-Mix substrate was ideal for achieving these results because it is composed of peat moss, perlite, vermiculite, and wetting agents, and has a fertilizer-based formulation. The greater capacity to retain air and water, combined with its good drainage characteristics, was ideal for acclimatizing strawberry cultivars; the physical characteristics of this substrate allowed the strawberry seedlings to not dehydrate.

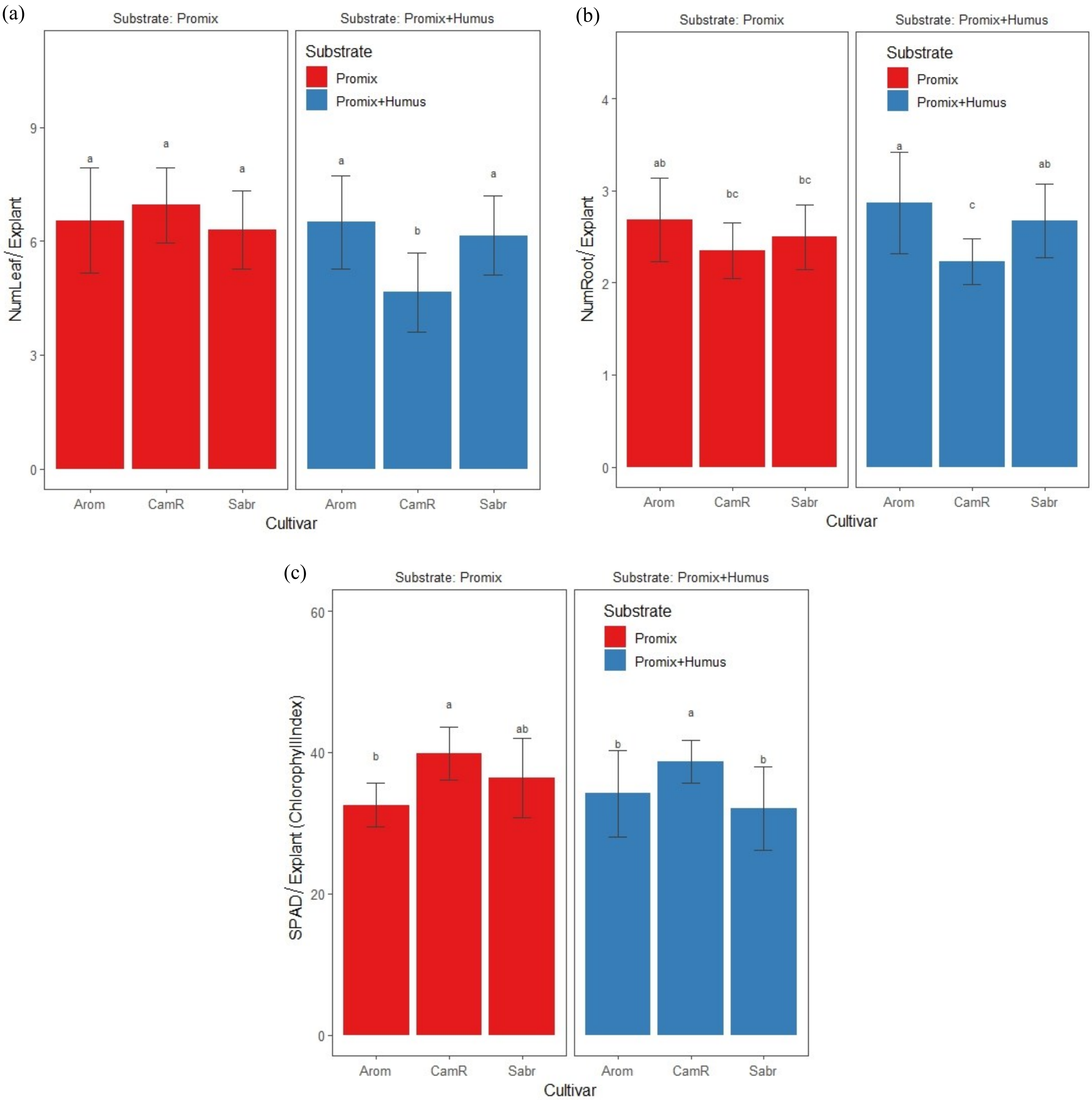
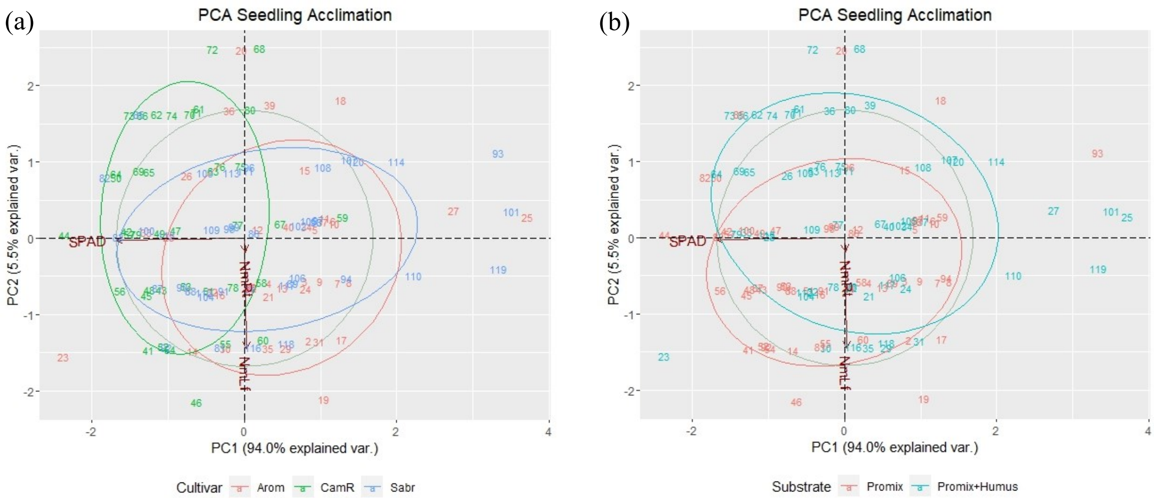


Figure 3. Acclimatization of strawberry cultivar seedlings. a) Number of leaves, b) number of roots, c) SPAD index. The data are presented as the means ± standard deviations, and different letters indicate significant differences in the parameters for p≤ 0.05 according to the Tukey test.



Arom (Aromas), CamR (Camino Real), and Sabr (Sabrina)
NmRt (number of roots), NmLf (number of leaves), SPAD (SPAD index)

Figure 4. Main components involved in the acclimatization of strawberry plants. a) Graph demonstrating the degree of association and divergence in the strawberry cultivars. b) Graph demonstrating the degree of association and divergence in the use of the types of the Pro-Mix and, Pro-Mix plus humus substrates.

Rooted plants are usually very sensitive to environmental changes, so success or failure will depend on the acclimatization process (CASTILLO, 2004). There are various techniques used in the acclimatization stage for strawberry seedlings, for example, using germination trays placed inside transparent containers and covered in such a way as to simulate a mini greenhouse, such as what was used in this study. During the acclimatization process, Félix-Hernández; López-López; Alvarado-Rodríguez (2017) applied an interesting strategy; placing the rooted shoots on a plastic base, simulating a floating hydroponic system, changing the water daily for 15 days, and then transferring them to a vermicompost and agrolitea-based substrate; at a ratio of 1:1. This method allowed the plants to acclimatize faster and without stress due to moisture loss.

In the different acclimatization methods, in addition to finding a suitable substrate, it is also important to provide the seedlings with a controlled environment so that they do not die due to dehydration. Similarly, during frequent irrigation with Hoagland's solution, 90% of the Camarosa cultivar was able to survive in the substrate at field capacity (HADDADI et al., 2010). On the other hand, Dhukate et al. (2021) obtained a survival of 95% in the acclimatization of the Sweet Charlie and Winter Dawn cultivars in a cocopeat substrate.

The amount of chlorophyll that is present in leaves can vary according to the cultivar, vegetative period, fertilization (KARELE, 2001), and water stress (BAUERLE et al., 2004). The chlorophyll index is an indicator of the nitrogen content in a plant. Several researchers recommend using at Minolta SPAD-502 chlorophyll meter for measuring the nutritional status of a plant in relation to its nitrogen content. A higher dose of nitrogen in the seedlings results in a greater photosynthetic rate, as reflected by higher SPAD index values. (RIBEIRO et al., 2015). Different works have reported chlorophyll SPAD indices for the purpose of diagnosing nitrogen levels in cultivars; for example, a range between 40.5 and 43.7 is ideal for sweet potato cultivation (COELHO et al., 2010). In the cultivation of hydroponic strawberry plants, Gómez (2011); registered SPAD index readings between 54.4 and 56.2; for 75 to 135 days after transplanting. Similarly, SPAD index values have been reported for another hydroponic strawberry crop, with 38.5 to 49.5 SPAD indices for young and old leaves, in different substrates (50% cocopeat and 50% perlite) (AHMADIZADEH; EBRAHIMI; EBRAHIMI, 2012).

In the present study, the Camino Real and Sabrina cultivars exhibited greater chlorophyll indices (39.8 and 36.4, respectively) when the Pro-Mix substrate was used, which is important for proper establishment of the substrate in the acclimatization stage. In a previous report (NERI et al, 2022) on the acclimatization of seedlings of the Aromas cultivar, the chlorophyll index when using a substrate based on compost + peat + humic acid, presented a value of 40.69 SPAD, this value is greater than that reported in the present investigation with Pro-Mix (32.5) or Pro-Mix plus humus (34.2) for the same cultivar. This could be related to the time at which the variable was evaluated, the state of the evaluated leaf (young leaf or adult leaf) or the substrate used.

For *ex vitro* acclimatization, the seedlings have well-developed leaves, since they will carry out photosynthesis and the plants have an energy source that allows them to generate more roots and thus be able to efficiently assimilate the nutrients present in the substrate. The chlorophyll content of leaves is closely related to the level of nitrogen that the plant absorbs and is a reliable indicator of the vegetative growth of the plants during the acclimatization stage (MIXQUITITLA et al., 2020). Therefore, when the nitrogen level is low, the photosynthetic activity is also low, and in some extreme cases, the plants may show symptoms of chlorosis in their leaves, which is a clear indicator of nitrogen deficiency. In the *ex vitro* acclimatization of the three strawberry cultivars, no chlorosis was observed in the leaves, which indicates that the SPAD values were adequate; therefore, the root system at the sixth week of acclimatization had the ability to efficiently absorb the nutrients present in the substrate.

CONCLUSION

We established an efficient *in vitro* method for the induction of adventitious shoots in three strawberry cultivars through the combined use of cytokinins. KIN (1.0 mg L^{-1}) + Zt (1.0 mg L^{-1}) was the best combination of cytokinins, since it was possible to induce the greatest number of shoots per explant in the strawberry cultivars, Sabrina (21 shoots), Aromas (18 shoots), and Camino Real (15 shoots). This combination of cytokinins was adequate because the explants did not produce calli in the three strawberry cultivars under study. Likewise, they promoted the development of the root system when transferred to MS media without growth regulators; therefore, medium was not required for growth or root induction. In the acclimatization phase, a 100% survival of the acclimatized plants was achieved. The Pro-Mix or Pro-Mix plus humus substrates did not significantly affect the number of leaves, number of roots, or SPAD index. The Pro-Mix substrate alone proved to be the best substrate for the acclimatization of the Camino Real strawberry cultivar, producing a greater number of leaves.

ACKNOWLEDGEMENTS

The APC was financed by the Vicerrectorado de Investigación, Universidad Nacional Toribio Rodríguez de Mendoza de Amazonas.

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