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Double-phase culture medium and plant growth regulators in the micropropagation of blackberries

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Abstract

Micropropagation is a technique that consists of producing disease-free and genetically uniform *in vitro* plants, which are produced in a shorter period of time than conventional propagation methods. The purpose of this study was to evaluate the effect of the consistency of the culture medium and the concentrations of BAP on the multiplication of sprouts, and concentrations of IBA on *in vitro* rooting of the Ébano and Tupy blackberry cultivars. The experiments were divided into three parts: In the first stage, culture media with solid, liquid, and double-phase texture were evaluated aiming at selecting the best culture medium in relation to quantity and quality of the sprouts. In the second stage, BAP concentrations were analyzed aiming at increasing the quantity of sprouts. In the third stage, IBA concentrations were evaluated aiming at optimizing root formation in the cultivars. The double-phase and liquid culture did not differ in the number of sprouts and leaves per explant and length of sprouts. The highest multiplication of sprouts on double-phase medium occurred with 5 μM L^{-1} BAP. Rooting was stimulated with the use of IBA and the best concentration was estimated at 1.1 μM IBA. The multiplication of Ébano and Tupy cultivars can be carried out in double-phase MS medium with 5 μM L^{-1} BAP and rooting in MS medium with 1.1 μM L^{-1} IBA.

Keywords: Double-phase culture medium; BAP; IBA; plant growth regulators; *Rubus*

Introduction

Tupy and Ébano are Brazilian blackberry cultivars released by the Embrapa Clima Temperado breeding program in 1981 and 1988, respectively (Raseira & Franzon, 2012). The 'Tupy' cultivar stood out for its productivity, quality, and fruit size. It was considered the most important blackberry in the world in 2010, being produced in large volumes in Mexico and exported in the form of fresh fruit to the United States, Canada, and Europe (Volk et al., 2013).

The propagation of the blackberry tree is usually done by root cuttings. However, it can be done by tissue culture through micropropagation, which aims at obtaining plants free of disease, genetically uniform, and produced more quickly than the conventional process (Villa et al., 2008).

Several micropropagation protocols for blackberry were found, however, the results obtained were very variable and changed according to the cultivar choice. For example, Schiehl (2020) used the Xingu cultivar (hybrid resulting from the cross between 'Tupy' and 'Arapaho'), and the most efficient concentration found for *in vitro* multiplication was 5 μM L^{-1} BAP. Whereas for Xavante cultivar, the highest number of sprouts was found with 15 μM L^{-1} BAP (Leitzke et al., 2010), and for the Chester Thornless cultivar, with a multiplication rate of 8.8 μM L^{-1} BAP + 0.9 μM L^{-1} IBA (indolebutyric acid) (Kefayeti et al., 2019).

Besides plant growth regulators, choosing the double-phase medium is also considered a strategy for increasing the number of sprouts for woody shrub species (Kozomara et al., 2008). The double-phase medium promotes greater growth of the aerial part of the regenerated sprouts (Martínez et al., 2012). Other studies have also been conducted with the double-phase medium in pear and apple rootstock, where it showed

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an increase in the *in vitro* multiplication rate of sprouts (Moraes et al., 2004) (Machado et al., 2004).

Therefore, due to the importance of this theme for micropropagation, the purpose of this study was to evaluate the effects of the consistencies in the *in vitro* culture media, the BAP concentrations in the multiplication of sprouts, and the concentrations of IBA in the *in vitro* rooting of the Ébano and Tupy cultivars.

Material and Methods

In this study, Ébano and Tupy blackberry cultivars supplied by Embrapa Clima Temperado in Pelotas - RS were used. The explants were obtained from plants grown *in vitro* on MS culture medium (Murashige & Skoog, 1962) with 5 μM L^{-1} BAP.

After the multiplication period, the experiments were initiated and divided into three stages for this study. In the first stage, the consistency of the MS culture medium was evaluated, being solid, liquid and double phase. In all media, 4 μM L^{-1} BAP was added. The flasks used in this phase had a 240 mL capacity and 30 mL of MS medium was added to the solid and liquid media. For the double-phase MS culture medium, 20 mL of solid MS medium and 10 mL of liquid MS medium were used. The design used was entirely random, with 4 repetitions and 2 flasks per plot. Each flask with 5 explants, totaling 24 flasks per cultivar. The treatments were arranged in a factorial arrangement (2x3), with 2 cultivars and 3 culture media.

In the second stage, the effect of six BAP concentrations (0.1, 2, 3, 4, and 5 μM L^{-1}) in double-phase MS medium was evaluated. The double-phase MS medium was prepared in 240 mL capacity flasks, where 20 mL of solid MS medium without plant growth regulators was added, and after solidification, 10 mL of liquid MS medium with the above-mentioned BAP concentrations was added. The design used was entirely random, with 4 repetitions and 1 flask per plot. Each flask contained 5 explants, totaling 24 flasks per cultivar. The treatments were arranged in a factorial arrangement (2x6), with 2 cultivars and 6 concentrations of BAP.

In the third stage, the effect of IBA (indole-3-butyric acid) on *in vitro* rooting, composed of five different IBA concentrations (0; 0.5; 1.0; 1.5; and 2.0 μM L^{-1}) in solid MS culture medium, was evaluated. The design used was also entirely random, with 4 repetitions and 1 flask per plot. Each flask contained 4 explants, totaling 20 flasks per cultivar. The treatments were arranged in a factorial arrangement (2x5), with 2 cultivars and 5 IBA concentrations.

For the preparation of all MS media, 30 g L^{-1} sucrose was added, solidified with 7 g L^{-1} agar (Vetec®) (except liquid MS medium), and the pH was adjusted to 5.8. The media were autoclaved at 121°C and 1 atm for 20 minutes. The flasks were transferred to a growth room at 25 ± 2°C, with an irradiance of 35 mmol m^{-2} s^{-1}, and a 16-hour photoperiod. The evaluations were performed 60 days after setting up the experiments. The variables analyzed in the multiplication stages were number of sprouts, number of leaves, length of sprouts (cm), and rooting percentage. In the rooting experiment, the variables analyzed were rooting percentage and root length between 0–0.5, 0.5 –1, and 1.0 –1.5 mm in diameter, total length (cm), and total volume (cm^3), obtained with the WinRhizo® software.

The data was submitted to analysis of variance, and the percentage data was transformed into arcsin square root (x + 0.5) for analysis. The comparison of the qualitative factors, consistency of culture medium, and cultivars means was performed by the Tukey test at 5% error probability. The comparison of quantitative factors and BAP and IBA concentrations, on the other hand, was performed by regression analysis. Statistical analyses of the experiments were performed using the software Assistat® Version 77 beta (2016).

Results and Discussion

In the first experiment, the consistency of solid, liquid, and double-phase culture medium was evaluated. There was no interaction between the factors for the variables number and length of sprouts, but there was an interaction for the number of leaves per sprout.

The cultivars differed in the number of leaves per sprout, with the Tupy cultivar showing higher results compared to the Ébano cultivar in double-phase and liquid MS culture media, however, no differences were found in solid culture medium (Table 1).

The number of sprouts was higher in the double-phase and liquid culture media, which did not differ and presented 8.6 and 10.2 sprouts per explant, respectively, while in the solid medium there were 4.8 sprouts per explant (Table 1). The growth of plants in the double-phase and liquid media was clearly higher (Figure 1).

Table 1. Number of new sprouts, number of leaves, and length of sprouts of Ébano and Tupy blackberry cultivars after cultivation on solid, double-phase, and liquid MS culture medium.

Cultivars	Solid	Double-phase	Liquid	Mean
		Number of sprouts		
Tupy	5.4	8.7	9.4	7.8 A
Ébano	4.4	8.5	11.0	7.9 A
Mean	4.8 b	8.6 a	10.2 a	
CV (%)		33.5		
		Number of leaves		
Tupy	7.6 aA	8.1 aA	8.8 aA	8.2
Ébano	8.7 aA	7.0 aB	6.9 bB	7.5
Mean	8.1	7.6	7.9	
CV (%)		14.1		
		Length of sprouts (cm)		
Tupy	2.2	3.1	3.0	2.8 A
Ébano	2.3	2.9	2.5	2.6 A
Mean	2.3 b	3.0 a	2.8 ab	
CV (%)		22.0		

*Means followed by the same capital letter in the column and lower case in the row do not differ significantly by the Tukey test at 5% probability.

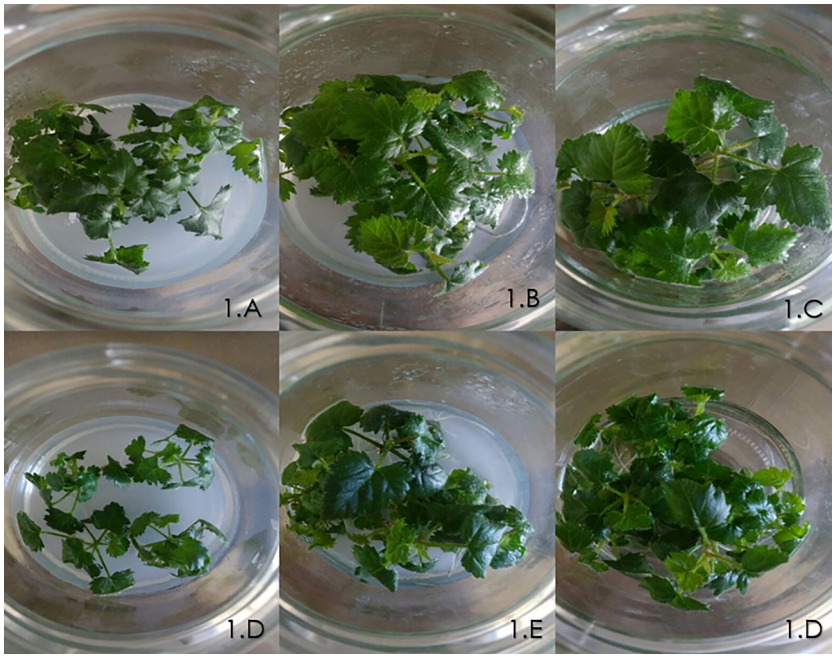

Figure 1. Tupy cultivar in solid (1.A), double-phase (1.B), and liquid (1.C) culture medium, and Ébano cultivar in solid (1.D), double-phase (1.E), and liquid (1.F) culture medium.

According to Pereira & Fortes (2003), the liquid MS medium has the greatest contact with the explants, providing an increase in the water and nutrients absorption when compared to other media. This greater contact between plants and culture medium that occurs in the liquid medium has the advantage of favoring the nutrient assimilation, height, and sprout multiplication rates.

Another advantage of the liquid MS medium is the ease of medium preparation and, especially, the cost reduction by not using agar. However, the liquid medium presents a greater difficulty for the fixation of plants, which cannot remain submerged in the medium, as it would cause their oxidation and death. Thus, the explants should be large enough so a part of them could remain outside of the medium and allow growth in the new sprouts. Another problem in liquid medium is the hyperhydricity phenomenon, which is aggravated in stationary liquid media.

The Xingu cultivar, despite presenting great superiority in growth in the liquid medium when compared to the solid medium, formed sprouts with a high level of hyperhydricity, compromising its quality and making its recommendation unfeasible (Schiehl et al., 2020). In the case of blackberry, this is a species sensitive to this physiological disorder, although the Ébano cultivar is more tolerant than other cultivars .

The double-phase culture medium becomes an interesting option to allow the fixation of explants and to have the advantage of the greatest absorption of nutrients of the liquid medium, as already verified with orchids, in

which the double-phase medium allowed to obtain a 2.5 times higher rate of multiplication of sprouts than the semi-solid medium after 90 days of cultivation (de Oliveira et al., 2013). The 'Marubakaido' apple rootstock showed higher number, height, and mass of sprouts on the double-phase medium compared to the semi-solid medium (Machado et al., 2004). In the micropropagation of lingonberries (*Vaccinium vitis-idaea* L.), the double-phase medium produced twice as many sprouts than the Zimmerman & Boome (1980) culture medium, with only the semi-solid phase (Mazurek & Siekierzyńska, 2018). However, not all species adapt to this technique, as verified with lisianthus (*Eustoma grandiflorum*), which did not develop in double-phase and liquid media (Kaviani & Bahari, 2019).

In the second experiment, Ébano and Tupy cultivars were evaluated for the effect of *in vitro* multiplication in double-phase MS culture medium, considering different concentrations of BAP. There was no interaction between the two factors evaluated for the number of sprouts (Figure 2.A) and number of leaves per explant (Figure 2.B), but there was an interaction for the length of sprouts (Figure 2.C). All the explants in this experiment had roots. The Ébano cultivar showed a higher number of sprouts than the Tupy cultivar, but it did not differ regarding the number of leaves (Table 2).

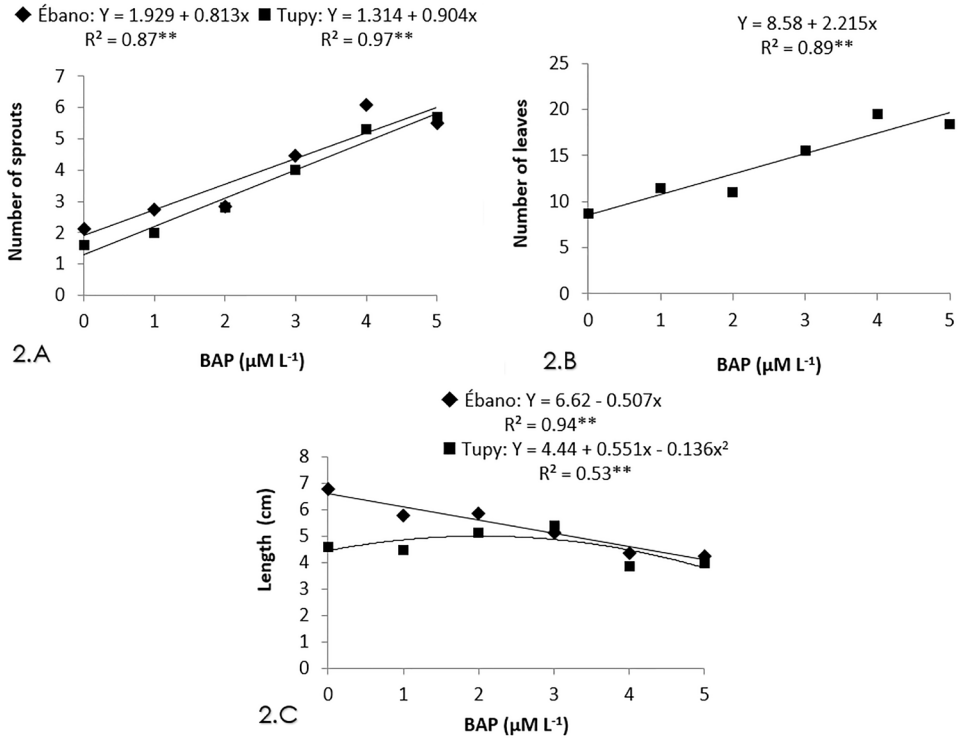

Figure 2. Number of new sprouts (2.A), number of leaves (2.B), and length (2.C) of the new Ébano and Tupy blackberry cultivars after cultivation on double-phase MS culture medium with different concentrations of BAP.

Table 2. Number of leaves per sprout and percentage of rooted explants of the Ébano and Tupy blackberry cultivars after cultivation in double-phase MS medium with different concentrations of BAP

Cultivars	Number of leaves	Rooting (%)
Tupy	13.8 a	100
Ébano	14.4 a	100
CV (%)	9.69	-

*Means followed by the same letter do not differ by Tukey test at 5% probability.

The number of sprouts and number of leaves of the Ébano and Tupy cultivars on double-phase MS medium was represented by a linear upward trend. With the increase in the cytokinin (BAP) concentration, there was an increase in the means of these variables (Figures 2.A and 2.B) up to a 5 µM L^{-1} concentration. This result was also found for the multiplication of the Xavante cultivar (Toledo & Biasi, 2018) and, according to Erig (2002), for the Tupy cultivar, a maximum induction of multiplication of sprouts was also observed with MS medium in the presence of BAP with linear upward trend concentrations at 2, 4, and 6 µM L^{-1}.

In the length variable (Figure 2.C) it was observed that with an increasing in BAP concentrations, there is a reduction in the length of the sprouts. This behavior was also found in other studies, such as the study by Villa et al.,

(2008), who evaluated the Ébano cultivar and obtained a decrease in the length of the sprouts with increasing concentrations of cytokinin (BAP). Cytokinins act by stimulating cell division, which promotes the proliferation of axillary and adventitious buds in explants (Wybouw & De Rybel, 2019). The increase in the number of developing sprouts generates a greater competition for nutrients and carbohydrates in the culture medium, causing these sprouts to grow less.

In the third experiment, the effect of IBA on the *in vitro* rooting of Tupy and Ébano cultivars in solid MS culture medium was evaluated. All variables showed significant interaction, and a quadratic behavior was observed in relation to the levels of IBA, regarding root lengths (Figure 3.A, 3.B, 3.C), total length (Figure 3.D), and total volume (Figure 3.E). By the regression equations obtained in this experiment, it was possible to estimate a 1.1 µM L^{-1} IBA average concentration that allowed a better length quality and maximum volume of roots (Figure 4). The quadratic behavior was also observed for the Tupy and Brazos cultivars, which had an increase in the number of roots with the increase in the concentration of naphthaleneacetic acid (NAA) up to the concentration of 1mg L^{-1} (Villa et al., 2008). This response is probably associated with endogenous levels of indole acetic acid (IAA), which is the key auxin in inducing root organogenesis, but whose increased concentration can cause the inhibition of root elongation. IBA, which was initially described as a synthetic auxin, is found endogenously in plants and converted to IAA by enzymatic action, with its distribution and conversion being considered as the factors responsible for the root system architecture (Overvoorde et al., 2010).

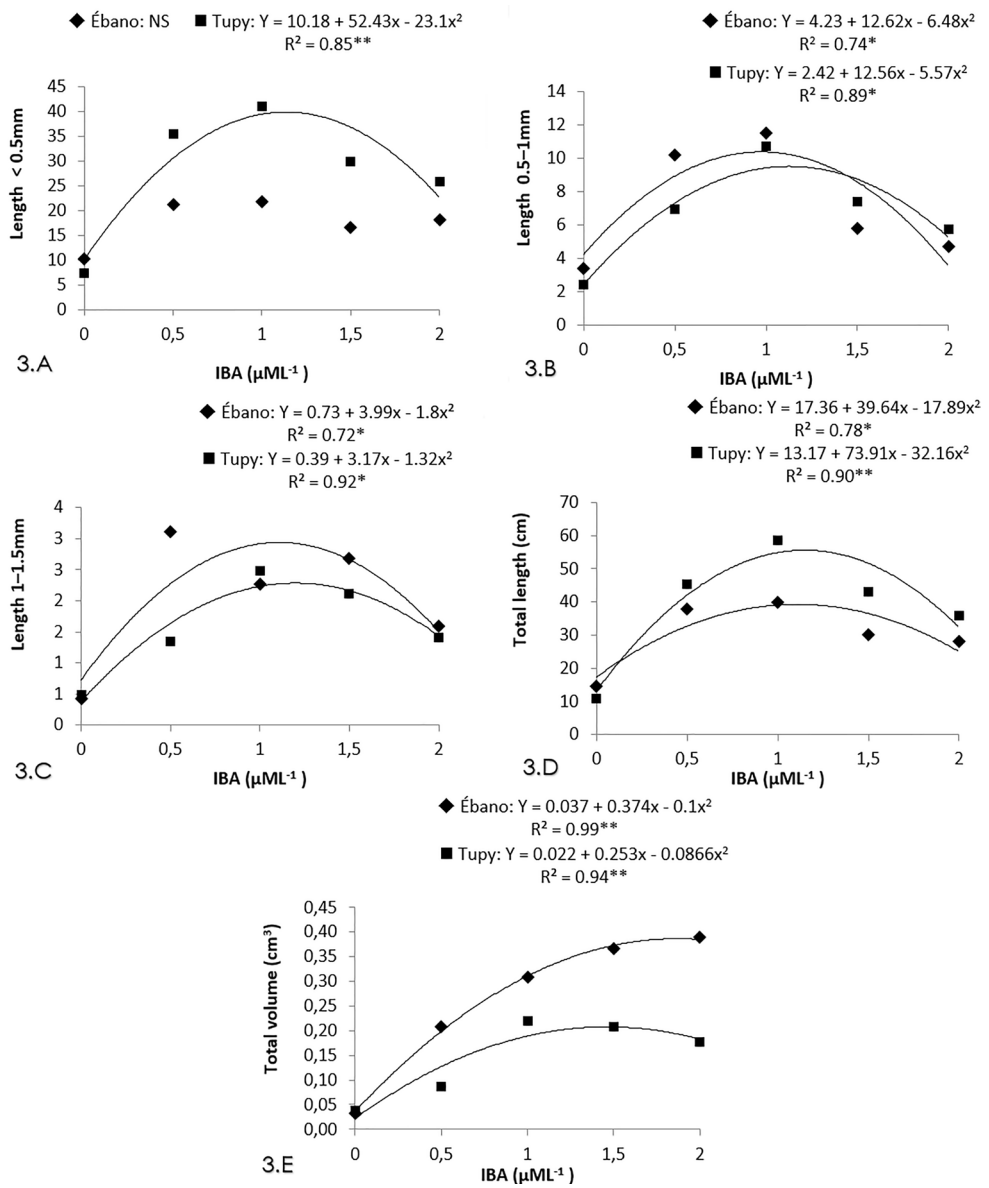

Figure 3. Root length with less than 0.5 mm in diameter (3.A), root length between 0.5 and 1.0 mm in diameter (3.B), root length between 1 and 1.5 mm in diameter (3.C), total root length (3.D), and total root volume (3.E) of Tupy and Ébano cultivars after cultivation on solid MS medium with different concentrations of IBA.

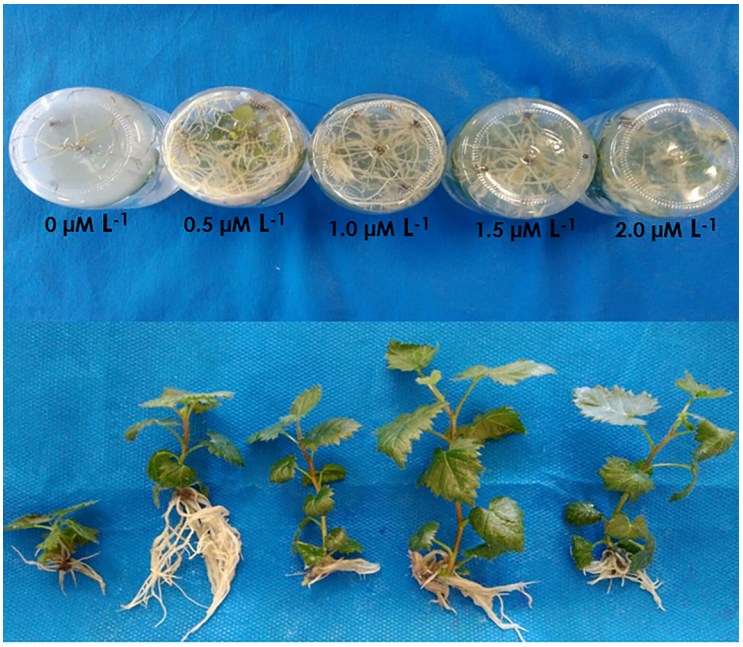

Figure 4. Tupy cultivar on MS culture medium with five different concentrations of IBA (0; 0.5; 1.0; 1.5; and 2.0 µM L^{-1}).

The Ébano cultivar showed a reduction in the number and length of roots formed with the application of IBA at 0.5 and 1µM concentrations, combined with dark periods of up to 6 days (Radmann et al., 2003). However, in this study, the authors did not evaluate higher concentrations of IBA and they also did not separate the roots according to their diameter. In the *ex vitro* rooting of the Xingu cultivar, the application of IBA in the form of rapid immersion of the sprouts' base in solutions of up to 9.8 Mm did not increase the volume and total length of roots, but promoted an increase in the length of roots with more than 1 mm in diameter (Schiehl et al., 2020).

Conclusion

Considering the results presented in this study, it was possible to conclude that the *in vitro* multiplication of the Tupy and Ébano blackberry cultivars can be performed in double-phase MS medium with 5 μML^{-1} BAP, and rooting in solid MS medium with 1.1 µM L^{-1} IBA.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.