FOLIAR ANATOMY AND IN VITRO GROWTH OF *Cattleya* AT DIFFERENT CONCENTRATIONS OF KEFIR, KNUDSON MEDIUM, AND SUCROSE

ANATOMIA FOLIAR E CRESCIMENTO IN VITRO DE Cattleya *EM DIFERENTES CONCENTRAÇÕES DE QUEFIR, MEIO KNUDSON E SACAROSE*

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ABSTRACT: Kefir is a probiotic used for human nutrition because of medicinal and nourishing properties. The purpose of this paper was to evaluate the foliar anatomy and *in vitro* growth of orchids cultivated at different concentrations of Knudson medium, kefir and sucrose. The treatment consisted of different culture media: 25% of Knudson (KD) salts + 75% of kefir (KF); 50% of KD + 50% of KF; 75% of KD + 25% of KF, volume per volume (v/v) at every possible combination with either 10 $g.L^{-1}$ or 20 $g.L^{-1}$ of sucrose. Two other treatments were done: one with 100% of Knudson salts added with 20 $g.L^{-1}$ of sucrose, and the other with kefir alone. The culture medium had the pH adjusted to 5.8 and was solidified with agar 7 $g.L^{-1}$. Forty mL of culture medium were distributed into 250 mL-vessels, which were then autoclaved at 120 °C for 20 minutes. The best results for the *in vitro* growth were obtained with 50% KD + 50% KF with 20 $g.L^{-1}$ of sucrose. The anatomical studies revealed that the 25% KD + 75% KF culture medium with 10 $g.L^{-1}$ and 20 $g.L^{-1}$ elicited a thicker foliar mesophile, being followed by 50% KD + 50% KF with 10 $g.L^{-1}$ of sucrose, and 75% KD + 25% KF with 20 $g.L^{-1}$ of sucrose.

KEYWORDS: Micropropagation. Orchids. Probiotic.

INTRODUCTION

Orchids are ornamental plants grown for the beauty and exoticness of their flowers. However, the conventional propagation of such plants presents a low yield. Tissue culture techniques for *in vitro* propagation and growth of orchids can be improved by the use of new substances for a more efficient micropropagation process.

The addition of complex mixtures to the culture medium, such as coconut water, malt extract, and banana juice, has been successfully used for different species since the beginnings of tissue culturing (LOEWENBERG; SKOOG, 1952), and the culture medium has been supplemented with amino acids, vitamins and growth regulators (GEORGE, 1993). The function of complex mixture, into culture medium, has been promoted growth of micropropagated plants (SILVA et al., 2005).

Kefir, a Slavic word for "well being", is a milk beverage containing lactobacilli, lactococci, yeasts and acetic bacteria. Known for its organoleptic properties and use in popular medicine, it has been used for thousands of years by the peoples of the Caucasus mountains. In human medicine, this probiotic has been used in the regulation of the intestinal microbiota, and as an immunomodulator with anti-carcinogenic activity (KUBO et al., 1992).

Recent studies demonstrated that kefir, when used at a 20% concentration, either autoclaved or not, induces the synthesis of phytoalexins in soy cotyledons, and also inhibits germination in uredioniospores of *Phakopsora pachyrhizi*, a fungus which cause Asian rust, in 87% and 95%, respectively (MESQUINI et al., 2007). The action of kefir in plant physiology and morphology is so far unknown. No such data were found in the literature.

The purpose of this paper was to evaluate the *in vitro* growth and foliar anatomy of orchids kept in a culture medium with different concentrations of Knudson medium, kefir and sucrose.

MATERIAL AND METHODS

This research was carried out at the José do Rosário Vellano University (UNIFENAS) Laboratory of Plant Biotechnology, in Alfenas/MG. The plants were obtained through seed germination from the self-fertilization of *Cattleya walkeriana* in Knudson (1922) culture medium, uniformized at the length of 1.0 cm for mounting the experiment.

Kefir was cultivated at the UNIFENAS Laboratory of Biology and Physiology of Microorganisms. Five grams of kefir were cultivated in 50 g of brown sugar for 1 liter of filtered water, and the nutritive solution was

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replaced every 24 hours, kept at room temperature, and cultivated for 15 days before the beginning of the experiment. Then, the grains were gently washed in running water, blended, sieved and added together with the culture medium according to the concentration of each treatment.

The treatments consisted of different culture media: 25% of Knudson salts (KD) and 75% of kefir (KF); 50% of KD and 50% of KF; 75% of KD and 25% of KF, volume per volume (v/v) with 10 $g.L^{-1}$ or 20 $g.L^{-1}$ of sucrose. Two additional treatments were also done: one, with 100% of Knudson salts, added with 20 $g.L^{-1}$ of sucrose; the other, composed by only kefir and water. For all the treatments, the culture medium had the pH adjusted to 5.8 and was solidified with 7 $g.L^{-1}$ of agar. Forty mL of culture medium were distributed into 250 mL-vessels, which were identified according with each treatment, and then autoclaved at 120 °C for 20 minutes. The cultivation was kept in a growth room with temperature of 24 ± 2 °C, constant photosynthetic photon flux densities of 36 µmol $m^{-2}s^{-1}$ and a 16-hour photoperiod.

After 180 days, the plants were evaluated with regard to: length of the aerial part and root system; number of roots and leaves; and fresh matter and dry matter. For foliar anatomy, the data were the following: mesophile thickness; thickness of the adaxial and abaxial epidermis; polar and equatorial diameters of the stomata under light microscope (LM), according to Kraus & Arduin (1997).

The design was entirely randomized (ERD), consisting of 8 treatments with 4 repetitions, and 4 plants per parcel. The Sisvar (FERREIRA, 1999) was used for the analysis of variance, and the means were compared by the Tukey test.

RESULTS AND DISCUSSION

The analysis of all the variables revealed a significant effect of the culture medium and the sucrose concentrations. The medium 75% KD + 25% KF, added with 20$g.L^{-1}$ of sucrose, provided a larger number of leaves (Table 1), followed by 50% KD + 50% KF with 10 $g.L^{-1}$ or 20$g.L^{-1}$ of sucrose. For the variables length of the aerial part and root system, the best result was obtained with 50% KD + 50% KF with 20 $g.L^{-1}$: 9.56 cm, and 4.82 cm, respectively (Table 1).

Table 1. Number of leaves (N leaves), length of the aerial part (LAP), length of the root system (LRS) in different culture medium and sucrose concentrations.

Medium	N leaves Sucrose ($g.L^{-1}$)		LAP (cm) Sucrose ($g.L^{-1}$)		LRS (cm) Sucrose ($g.L^{-1}$)	
	10	20	10	20	10	20
25%KD + 75%KF	7.76 Cb	8.66 Ca	2.91 Db	4.00 Ba	3.51 Bb	3.84 Ba
50%KD + 50% KF	9.47 Ba	9.97 Bb	2.83 Db	9.56 Aa	2.20 Cb	4.82 Aa
75%KD + 25%KF	8.49 Bb	13.10 Aa	2.87 Db	3.52 Ca	3.48 Ba	1.98 Db
Knudson*	-	7.00 D	-	2.81 D	-	2.42 C
Kefir* **	-	6.78 E	-	2.82 D	-	3.49 B

Means followed by the same capital letter, on the vertical axis, and small letter, on the horizontal axis, are not different from one another by the Tukey test with 5% of probability. * = additional treatment; KD = Knudson ; KF = kefir; ** = kefir + agar treatment.

The largest growth of the aerial part in the in vitro cultivation of orchids is an important factor, since it is a relatively slow-growth plant, which takes more time in laboratory conditions. Complex organic additives, although obtained from natural products with indefinite composition, really enrich the culture medium, and may be added to the medium to elicit a better response in the growth pattern (TORRES et al., 2001).

Sucrose is one of the main factors involved in the in vitro rooting. This paper shows that the increase in sucrose concentration, from 10 $g.L^{-1}$ to 20 $g.L^{-1}$, generally induced a larger growth of the root system. Another remarkable factor was the additional treatment with only kefir and agar, which also induced a satisfactory growth of the root system: length of approximately 3.5 cm. This performance may be related to the concentration of sugars, which may reach 60 g of sucrose per kilogram of kefir (ZOURARI; ANIFANTAKIS, 1988). Similarly, the number of roots increased with the increase in the sucrose concentration in the culture medium (Table 2). More roots were observed in the culture media composed of 50% KD + 50% KF or 75% KD + 25% KF. Debergh (1988), however, states that the reduction, or even complete elimination of sucrose from the medium should be used to facilitate the passage of the plants from the heterotrophic to the autotrophic stage in the acclimatization phase.

Table 2. Number of roots (N roots), plant fresh matter (PFM), plant dry matter (PDM), in different culture medium and sucrose concentrations.

Medium	N roots Sucrose (g.L^{-1})		PFM Sucrose (g.L^{-1})		PDM Sucrose (g.L^{-1})	
	10	20	10	20	10	20
25%KD + 75%KF	5.33 Bb	6.37Ba	0.54 Db	0.72 Ba	0.034 Cb	0.055 Ba
50%KD + 50%KF	6.34 Ab	6.97 Aa	0.66 Cb	0.87 Aa	0.038 Cb	0.078 Aa
75%KD + 25%KF	5.26 Bb	7.26 Aa	0.60 Cb	0.65 Ca	0.035 Ca	0.032 Ca
Knudson*	-	3.84 D	-	0.50 D	-	0.036 C
Kefir* **	-	4.73 C	-	0.29 E	-	0.018 D

Means followed by the same capital letter, on the vertical axis, and small letter, on the horizontal axis, do not differ from one another by the Tukey test at 5% probability. * = additional treatment; KD = Knudson; KF = kefir; ** = treatment with only kefir and agar.

The kefir grain is the best initiator of its own growth. It consists of a 0.3-3.5 diameter gel matrix with an approximate composition of 800-900 g/Kg of water, 2 g/Kg of lipids, 30 g/Kg of proteins, 60 g/Kg of sugars, and 7 g/Kg of ashes (ZOURARI; ANIFANTAKIS, 1988; GARROTE, 2006), although changes in concentrations may occur in accordance with the culture medium of the kefir grains. Historically, kefir seems to be milk fermented by kefir grains or clusters. The autoclaving process at 110 °C for 20 minutes inactivates bacteria and yeasts (MAINVILLE et al., 2001). Kefir proprieties, in this context, to added lipids, proteins, sugars and ashes, into the culture medium, probably this complex mixture improved growth of *in vitro* culture.

More fresh matter and dry matter of in vitro-cultivated orchids were yielded by the 50% KD + 50% KF culture medium with 20 g.L^{-1} of sucrose. This fifty-fifty combination of Knudson salts and kefir probably favored plant growth. According to George (1993), the growth and morphogenesis of micropapagated plantlets can be improved with the addition organic compounds into the culture medium. In plants, such substances are required by cells as metabolic catalysts.

Moreira et al. (2008) related kefir, cultivated into brown sugar medium, as mixture of carbohydrate: glucose (40%), rhamnose (24%), galactose (10%) and arabinose (26%). Probably, this mixture of carbohydrates, produced from aqueous fermentation of kefir, and combination with Knudson salts was responsible for the better performance of orchids plants micropropagated.

The thickness of the adaxial and abaxial epidermis was positively affected by the use of kefir in the culture medium (Table 3). The best result was obtained with culture medim composed by kefir and agar. The interaction of the 25% KD + 75% KF culture medium with 10 g.L^{-1} and 20 g.L^{-1} of sucrose induced a good performance of the variable thickness of the mesophile and leaf (Tables 3 and 4), followed by the treatments with 50% KD + 50% KF with 10 g.L^{-1} of sucrose, and 75% KD + 25% KF with 20 g.L^{-1} of sucrose.

Table 3. Thickness of the adaxial (Esp Adaxial) and abaxial (Esp Adaxial) epidermis, and of the foliar mesophile (Esp Mesofilo), in different culture medium and sucrose concentrations.

Medium	Esp Adaxial (µm) Sucrose (g.L^{-1})		Esp Abaxial (µm) Sucrose (g.L^{-1})		Esp Mesofilo (µm) Sucrose (g.L^{-1})	
	10	20	10	20	10	20
25%KD + 75%KF	20.60 Ba	20.60 Ba	10.03 Bb	10.06 Ba	530.52 Aa	494.24 Ab
50%KD + 50%KF	17.82 Cb	20.60 Ba	10.03 Ba	13.03 Ba	510.66 Ba	439.75 Cb
75%KD + 25%KF	20.45 Ba	16.33 Cb	10.03 Ba	10.06 Bb	459.47 Cb	485.00 Ba
Knudson*	-	20.60 B	-	10.30 B	-	450.60 D
Kefir* **	-	37.12 A	-	13.20 A	-	450.40 D

Means followed by the same capital letter, on the vertical axis, and small letter, on the horizontal axis, do not differ from one another by the Tukey test at 5% probability. * = additional treatment; KD = Knudson; KF = kefir; ** = treatment with only kefir and agar.

The micropropagated plants exhibited leaves with differentiated aspects, such as smaller leaf thickness, large intercellular spaces, and hypofunctional stomata, when compared with plants which were grown in natural environments (PIERIK, 1990; CAPELLADES et al., 1990). On the other side, the better organization and larger thickness of the foliar mesophile (KHAN et al., 2003; SERRET et al., 1997) reduce losses in the acclimatization phase (SERRET et al., 1997). A

better organization and larger thickness of the mesophile were observed in culture media containing kefir when compared with the anatomical development of plants cultivated exclusively in Knudson medium (Figure 1,A and B). This response may be related to the increase of osmotic pressure in the culture medium, thus regulating the entrance of water into the plant cell.

The effect of osmotic stress caused by elevated levels of sucrose, fructose and mannitol or by increased levels of gelling agents that decreased water potential both resulting in the reduction of hyperhydricity as reported by Debergh (1983) and Ziv (1990). Kefir exhibited positive effects on the orchid grown *in vitro* by improved plant development. In an earlier study (MILLS; BENZIONI, 1992), descreased water potencial was reported to stimulate leaf growth and wax deposition on leaf surface of leaves of jojoba seedlings grown *in vitro* and thus reduced water loss under ambient conditions (MILLS et al., 1997).

The equatorial and polar stomata diameters were influenced by the culture media and sucrose concentration. The culture media composed of 25% KD + 75% KF and 50% KD + 50% KF, both added with 10 or 20 $g.L^{-1}$ of sucrose, were responsible for the largest diameters (Table 4).

Table 4. Foliar thickness (Esp Foliar), equatorial (D equatorial) and polar (D polar) diameters of the stomata in different culture medium and sucrose concentrations.

Medium	Esp Foliar(µm) Sucrose ($g.L^{-1}$)		D equatorial (µm) Sucrose ($g.L^{-1}$)		D polar (µm) Sucrose ($g.L^{-1}$)	
	10	20	10	20	10	20
25%KD + 75%KF	550.55 Aa	548.30 Aa	27.41 Bb	28.18 Aa	29.22 Ba	29.24 Ba
50%KD + 50%KF	532.42 Ba	470.49 Db	27.34 Bb	26.47 Cb	32.71 Aa	32.67 Aa
75%KD + 25%KF	492.30 Cb	531.84 Ba	27.68 Bb	27.99 Ba	29.71 Bb	29.42 Ba
Knudson*	-	480.98 C	-	27.33 B	-	25.36 C
Kefir* **	-	470.54 D	-	26.10 C	-	30.28 B

Means followed by the same capital letter, on the vertical axis, and small letter, on the horizontal axis, do not differ from one another by the Tukey test at 5% probability. * = additional treatment; KD = Knudson; KF = kefir; ** = treatment with only kefir and agar.

The reduced stomatal control of plants *in vitro* is reported as one of the main causes of water loss during acclimatization (KHAN et al., 2003; CAPELLADES et al., 1990; SCUIUTTI; MORINI, 1995). The more elliptical stomata, i.e., those with the polar diameter longer than the equatorial one, are reported as the most functional (KHAN et al., 2003; CAPELLADES et al., 1990). Such positive relation can be evidenced (Table 4) in the treatments with kefir alone, 50% KD + 50% KF added with 10 or 20 $g.L^{-1}$ of sucrose. The relation was negative with the Knudson medium alone.

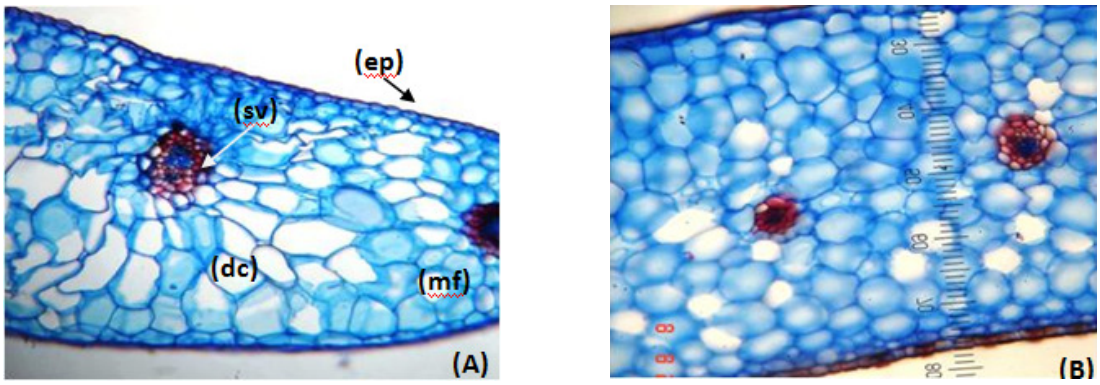

Figure 1. Foliar anatomy of plants cultivated *in vitro* in Knudson medium (A) and 25%KD + 75%KF (B); vascular system (sv); foliar mesophile (mf), epidermis (ep) and cell disorders (dc).

CONCLUSION

The use of kefir in *in vitro* orchid micropropagation promotes more growth, organization and thickness of foliar tissues, and may become a viable complex organic additive in the making of culture media.

RESUMO: O quefir é um probiótico utilizado na alimentação humana por suas características medicinais e nutricionais. O presente trabalho teve por objetivo verificar a anatomia foliar e crescimento *in vitro* de orquídea em meio de cultura com diferentes concentrações do meio Knudson, quefir e sacarose. Os tratamentos foram compostos por diferentes meios de cultura compostos pelas concentrações de 25% dos sais do Knudson (KD) e 75% de quefir (KF); 50% de KD e 50% de KF; 75% de KD e 25% KF, volume por volume (v/v), em todas as combinações possíveis com 10 ou 20 $g.L^{-1}$ de sacarose. Foram realizados também dois tratamentos adicionais um com 100% dos sais do Knudson, acrescido de 20 $g.L^{-1}$ de sacarose e outro composto somente por quefir. O meio de cultura teve o pH ajustado para 5,8 e solidificado com 7 $g.L^{-1}$ de agar. Foram distribuídos 40 mL de meio de cultura em frascos de 250 mL de conteúdo, os quais foram autoclavados a 120 °C por 20 minutos. O meio de cultura composto por 25% KD + 75% KF com 10 e 20 $g.L^{-1}$ de sacarose proporcionou melhor desempenho nos estudos anatômicos, sendo seguidos pelos tratamentos 50% KD e 50% KF com 10 $g.L^{-1}$ de sacarose e 75% KD e 25% KF acrescido de 20 $g.L^{-1}$ de sacarose, apresentando maiores espessuras de mesofilo foliar.

PALAVRAS-CHAVE: Micropropagação. Orquídeas. Probiótico.

REFERENCES

CAPELLADES, M.; FONTARNAU, R.; CARULLA, C. DEBERGH, P. Environment influences anatomy of stomata and epidermal cells in tissue culture *Rosa multiflora*. **Jornal of the American Society for horticultual Science,** Alexandria, v. 115, n. 1, p. 141-145, Jan. 1990.

DEBERGH, P. Effect of agar brand and concentration on the tissue culture medium. **Physiology Plant**, v. 59, p. 270-276, 1983.

DEBERGH, P. Improving mass propagation of in vitro plantlets. In: KOZAI, T. (Ed)**. Horitculturae in high techonology era. International Symposium on high techonology in protected cultivation.** Tokio, 1988. p. 45-47.

FERREIRA, D. F. **SISVAR 4. 3 – Sistema de análises estatísticas**. Lavras: UFLA, 1999.

GARROTE, G. L. et al. Rapid discrimination of lactobacilli isolated from *kefir* grains by FT-IR spectroscopy. **International Journal of Food Microbiology**, v. 111, n. 3, p. 280-287, 2006.

GEORGE, E. F. (Ed.) **Plant propagation by tissue culture.** pt. 1. The technology. 2. (Ed.). Edington: Exegetics, 1993. 574 p.

KHAN, S. V.; KOZAI, T.; NGUYEN, O. T.; KUBOTA, C.; DHAWAN, V. Growth and water relations of *Paulownia fortunei* under photomixotrophic and photoautotrophic conditions. **Biologia plantarum**, Copenhagen, v. 46, n. 2, p. 161-166, 2003.

KNUDSON, L. Nonsymbiotic germination of orchid seeds. **Botanical Gazette**, Chicago, v. 73, n. 1, p. 1-25, 1922.

KRAUS, J. E.; ARDUIM, M. **Manual básico de métodos em morfologia vegetal.** Rio de Janeiro: Seropédica, 1997. 198p.

KUBO, M.; ODANI, T.; NAKAMURA, S. Pharmacological study on kefir – a fermented Milk product in Caucasus antitumor activity. Yakugaku Zasshi, Tokyo, v. 12, p. 489-495, 1992.

LOEWENBERG, J. R.; SKOOG, F. Pine tissue cultures. **Physiologia Plantarum**, v. 5, p. 33-36, 1952.

MAINVILLE, I.; MONTPETIT,D.; DURAND, N.; FARNWORTH, E.R. Deactivating the bactéria and yeast in Kefir using heat treatment and high pressure. **International Dairy Journal,** Edmonton, v. 11, n. 1 e 2, p. 45-49, 2001.

MILLS, D.; BENZIONI, A. Effect of NaCl salinity on growth anddevelopment of clones: I. Nodal segments grown *in vitro*. **Journal Plant Physiology,** v. 139, p. 737–741, 1992

MILLS, D.; WENKART, S.; BENZIONI, A. Micropropagation of Jojoba. In: BAJAJ, Y.P.S. (ed). **Biotechnology in Agriculture and Forestry Vol. 40**. Springer-Verlag, Berlin, 1997. p. 370-393.

MESQUINI, R. M.; SCHWAN-ESTRADA, K. R. F.; BONALDO, S. M.; NASCIMENTO, J. F.; BALBI-PENA, M. I. Efeito de produtos naturais na indução de fitoalexinas em cotilédones de soja e na germinação de urediniósporos de Phakopsora pachyrhizi. **Revista Brasileira de Agroecologia**, Cruz Alta, v. 2, n. 2, p. 1091-1094, 2007.

MOREIRA, M. E. C.; SANTOS, M. H.; PEREIRA, I. O.; FERRAZ, V.; BARBOSA, L. C. A.; SCHNEEDORF, J. M. Atividade antiinflamatória de carboidrato produzido por fermentação alcoólica de quefir. **Química Nova**, v. 31, n. 7, p. 1738-1742, 2008.

PIERIK, R. L. M. **Cultivo *in vitro* de las plantas superiores**. Madrid: Mundi-prensa, 1990. 326 p.

SCUIUTTI, R.; MORINI, S. Water loss and photosynthesis of plum plantlets by relative humidity during rooting in vitro. **Journal of Horticultural Science**, Ahsford, v. 70, n. 2, p. 221-228, 1995.

SERRET, M. D.; TRILLAS. M. I.; MATAS, J.; ARAUS, J. L. The effect of different closure types, light and sucrose concentrations on Carbon isotope composition and growth of Gardênia jasminoides plantlets during micropropagation and subsequent acclimatization ex vitro. **Plant Cell, Tissue and Organ Culture**, Dordrech, v. 47, n. 3, p. 217-230, 1997.

SILVA, E. F.; PASQUAL, M.; PAIVA, P. D. O; SILVA, A. B.; NOGUEIRA, D. A. Polpa de banana e vitaminas do meio MS no cultivo in vitro de orquídea. **Plant cell Culture and Micropropagation**, Lavras, v. 1, n. 1, p. 8-12, 2005.

TORRES, A. C.; BARBOSA, N. V. dos REIS.; WILLADINO, L.; GUERRA, M. P.; FERREIRA, C. F.; PAIVA, S. A. V. de. **Meio e condições de incubação para cultura de tecidos de plantas.** Brasília: EMBRAPA-CNPH, 2001. (EMBRAPA-CNPH. Circular Técnica).

Ziv, M. Vitrification: morphological and physiological disorders in *in vitro* plants. In: DEBERGH, P. & ZIMMERMANN, R.H. (eds). **Micropropagation**. Kluwer Academic Publishers Dordrecht, 1990. p. 45-69.

ZOURARI, A.; ANIFANTAKIS, E. M. Lê Kefir caracteres physico-chimiques, microbiologiques et nutritionnels. **Thechnologie de production**, v. 68, p. 373-392, 1988.