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In vitro organogenesis in tomato cultivars is enhanced by gas exchange and application of ultrasound

A organogênese *in vitro* de cultivares de tomate é aumentada pela promoção das trocas gasosas e ultrassom

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ABCTRACT
The success of *in vitro* regeneration protocols is dependent of biological, chemical and physical factors. The manipulation of the microenvironment by enhancing gas exchange and ultrasound are physical improvements that potentially contribute to optimize *in vitro* responses. The present study evaluated the effect of gas exchange, by natural ventilation, on *in vitro* germination and further regeneration competence of explants exposed to sonication. For this, three tomato genotypes 'Moneymaker', 'NCEBR-2' and 'Santa Clara'. Cotyledonary and hypocotiledonary explants were subjected to ultrasound times (0, 3, 6, and 9 seconds) in order to figure out its influence on morphogenesis and regeneration. The results appoint the higher that gas exchange increased morphogenic growth responses in all the genotypes with a significant increase in cotyledon area and hypocotyl length in germination and biomass accumulation. The sonication time influenced the number of shoots higher than 0.5 cm and number of leaflets, showing an interaction between sonication and sealing type, highlighting the effect of exposure time to sonication on morphogenesis. In this study, we show for the first time the stimulation of organogenesis by the interaction of physical factors in *in vitro* culture: the use of high quality explants, obtained by enhanced gas exchange and the application of ultrasound. We suggest that these factors significant increase the quantity of organogenesis and reducing the time consumed in the *in vitro* process, using simple, reliable and cheap treatments as gas exchange facilitators caps and ultrasound.

Key words: gas exchange, plant regeneration, ultrasound, micropropagation

RESUMO
O sucesso dos protocolos de regeneração *in vitro* dependem de fatores biológicos, químicos e físicos. A manipulação do microambiente, através da promoção das trocas gasosas e o ultrassom, são fatores físicos que potencialmente contribuem na otimização das respostas *in vitro*. O presente estudo avaliou o efeito das trocas gasosas, por ventilação natural, na germinação *in vitro* e na competência regenerativa adicional de explantes expostos à sonicação. Para isso, três genótipos de tomate 'Moneymaker', 'NCEBR-2' e 'Santa Clara', ambos explantes cotilédones e hipocotiledonares foram submetidos a tempos de ultrassom (0, 3, 6 e 9 segundos), com o objetivo de avaliar sua influência na morfogênese e regeneração. Os resultados apontam que as trocas gasosas aumentam as respostas morfogênicas em todos os genótipos, com aumento significativo na área cotiledonar e no comprimento do hipocótilo ou mesmo na germinação e no acúmulo de biomassa. O tempo de sonicação influenciou o número de brotações acima de 0,5 cm e o número de folíolos, mostrando uma interação entre sonicação e tipo de selagem, destacando o efeito do tempo de exposição à sonicação na morfogênese. Neste estudo, mostramos pela primeira vez a estimulação da organogênese pela interação de fatores físicos na cultura in vitro: o uso de explantes de alta qualidade, obtidos com o auxílio das trocas gasosas, aprimorando com a aplicação de ultrassom. Sugerimos que esses fatores aumentem significativamente a quantidade de organogênese e reduzam o tempo consumido no processo *in vitro*, utilizando tratamentos simples, confiáveis e baratos como é o caso dos tampões facilitadores de troca gasosa ou mesmo de ultrassom.

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Palavras-chave: troca gasosa, regeneração de plantas, ultrassom, micropropagação

1 INTRODUCTION

The success of *in vitro* regeneration protocols is dependent on a number of factors among them, the genotype, age and vigor of mother plants, size and orientation of the explants in the medium (Collonier *et al.*, 2001; Costa *et al.*, 2008; Otoni *et al.*, 2003), and *in vitro* headspace gas composition (Kozai, 2010; Saldanha *et al.*, 2014). Recent studies suggests that *in vitro* gas composition is a key factor for obtaining high-quality seedlings, enabling more responsive and vigorous plantlets (Batista *et al.*, 2017; Bhatia and Sharma, 2015; Kiferle *et al.*, 2014; Saldanha et al., 2014). Changes in gas concentration in the *in vitro* environment, *e.g.*, decreasing relative-humidity and enhancing gas exchange, generally induces growth and morphogenesis of several species in different culture systems (Kiferle et al., 2014; Saldanha *et al.*, 2012; Saldanha et al., 2014). On the other hand, higher relative-humidity inside the flasks results in lower plant transpiration, low rates of water and nutrients uptake and higher respiration rates, which reduces growth rates of the plants (Kozai *et al.*, 1997). Among the strategies to decrease humidity and increase gas exchange, the use of special lids that allow gas exchange has been shown to be an efficient, low-cost and easy procedure (Buddendorf-Joosten and Woltering, 1996; Saldanha et al., 2012). Other interesting physical treatment is ultrasound, a widely used tool for the manipulation of plant and animal cells and organs. Following exposure to non-lethal levels of ultrasound, many plants exhibit altered characteristics at different organizational levels (Ananthakrishnan *et al.*, 2007; Dong *et al.*, 2002). This effect may be related to the cavitation waves, and further violent collapse of bubbles, generating high pressure and temperature. This could cause localized rupture of plasma membrane and lead to absorption of substances dissolved into the solution where cells are, with the subsequent restoration of membrane integrity. According to Safari *et al.* (2012) the physiological effects of ultrasound directly affect cell wall and plasma membrane.

Plants can respond to mechanical stress and injury. They can induces biochemical and molecular changes often associated with induced resistance mechanisms (Benikhlef *et al.*, 2013). For instance, wounded plants produce reactive oxygen species (ROS) undergo changes in lignifications [Singh *et al.* (2016) and references therein], jasmonic acid and other hormones or wound signals (Xia *et al.*, 2015) and exhibit changes in gene expression (Turpaev, 2002) that are associated with induced defense reactions. Ultrasonic treatment was reported to cause reversible inhibition of DNA, RNA and protein synthesis in *Pisum sativum* root meristem cells (Furusawa and Kondo, 2016). Ultrasonic irradiation significantly stimulates protein synthesis in plant cells and protoplasts and affects plasma membrane permeability (Dong et al., 2002). Also causes changes in the less stable, extended form of nucleolar chromatin (Elsner and Lindblad, 1989). In *in vitro*

cultivation, ultrasound is used to obtain the largest number of branches in the genetic transformation procedure known as Sonication-assisted *Agrobacterium*-mediated genetic transformation (SAAT) (Koetle *et al.*, 2017). Ananthakrishnan et al. (2007) stimulated multiple shoot regeneration from recalcitrant cotyledon explants from seeds before germination of commercial squash (*Cucurbita pepo* L.) that was the first demonstration that ultrasound exerts morphogenic influence by surface removal, however the technique has been caused hyper hydratation of the explants. The association of *in vitro* cultures with gas exchange and ultrasound to make regeneration optimization *in vitro* cultures can be helpful. Indeed, Chopra *et al.* (2012) achieved a higher number of regenerated branches in lentil cultivars when the sonication was used. These result were achieved in both leaves and roots of primary transformants recovered on kanamycin containing selection media confirmed the expression of transgene.

The Solanaceae is highlighted and considered as a model for the *in vitro* tissue culture studies (Bebeli and Mazzucato, 2008; Menda *et al.*, 2013). Several studies were designed to establish protocols and optimize the cultivation of these species aiming the application in other families. Currently the tissue culture Solanaceae is widely used to evaluate aspects of the regeneration process and applications such as transgenesis (Fuoco *et al.*, 2013; Pandey *et al.*, 2014; Setamam *et al.*, 2014; Wang *et al.*, 2013; Weinhold *et al.*, 2013). The transgenesis associated with a plant model, such as tomatoes, enables to study the gene expression, changes in plant metabolism and silencing of key enzymes in the primary metabolism (Sienkiewicz-Porzucek *et al.*, 2010), development of the reproductive body (Carrari and Fernie, 2006), cell wall and secondary growing (van der Merwe *et al.*, 2010), ecophysiological interactions (Prudent *et al.*, 2011) and responses to stresses (Johnson *et al.*, 2003).

In general, studies involving *in vitro* culture and genetic transformation of tomato and other species rely on juvenile explants, frequently established by *in vitro* germination (Collonier et al., 2001; Costa et al., 2008; Otoni et al., 2003). The present study evaluated the effect of gas exchange *in vitro* associated with the sonication in plant regeneration. For this, commercial porous membranes were used in the *in vitro* germination and growth seedlings for further source of explants. Cotyledonary and hypocotyledonary segments from different tomato genotypes were subjected to ultrasound in order to figure out the influence of natural ventilation and ultrasound on their morphogenetic and regenerative competence.

2 MATERIALS AND METHODS

2.1 EFFECT OF GENOTYPE AND GAS EXCHANGE IN THE TOMATO IN VITRO SEEDLINGS

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Seeds of the tomato 'Moneymaker', 'NCEBR-2' and 'Santa Clara' were surface-sterilized by immersing in 70% (v/v) ethanol for one minute, followed by 20 minutes in a solution of NaOCl 1.25% (v/v) chlorine, plus two drops of Tween 20^{TM} and rinsing in sterile deionized water three succesive times. Thereafter, the explants were transferred to flasks containing 40 mL of medium. The culture medium consisted of half-strength MS salts (Murashige and Skoog, 1962), Nitsch vitamins (Nitsch, 1969), 0.1 g L^{-1} myo-inositol (Sigma-Aldrich Chemical Co, Darmstadt, Germany, part number G1914), 20 g L^{-1} sucrose, 6.5 g L^{-1} agar Merck (Merck KGaA, Darmstadt, Germany, part number 232-658-1) at pH 5.8 ± 0.1. The experiment was conducted in a growth chamber under 16-hour photoperiod, temperature of 26 ± 2ºC under an irradiance of 37 ± 4 µmol m^{-2} s^{-1}.

The experimental design was completely casualized in a factorial 3 x 4 x 2, with 3 genotypes ('Moneymaker', 'NCEBR-2' and 'Santa Clara'"), 4 sealing methods [plastic polyvinyl chloride (PVC), rigid polypropylene caps (PP), PP containing one (1M) or two (2M) MilliSeal PTFE membranes] and 2 explants (hypocotyl and cotyledon segments), with four replicates, each replicates represented by a flask with 20 seeds. The morphogenesis was evaluated after 15 days of culture by the analysis of the variables cotyledon area (cm^2) and hypocotyl length (cm).

2.2. INFLUENCE OF ULTRASOUND ON THE IN VITRO REGENERATION OF EXPLANTS GENERATED IN HIGHER LEVELS OF GAS EXCHANGE

The response to ultrasound was assessed in explants derived from *in vitro* germinated seedlings in flasks with rigid polypropylene caps (1M or 2M) or caps (PP). Thus, in this experiment, we not analyzed the explants formed in flasks sealed with PVC plastic, because this explants are so fragile, that this can't resist or survive after application of ultrasound.

Tomato cotyledons and hypocotyls of 'Santa Clara' from different sealing systems: PP, 1M, and 2M were exposed to different times of ultrasound: 0, 3, 6 and 9 seconds, using a sonicator (Ultrasonic cleaner, Bransonic model B1210E-Mt, 80W, Branson Co. Danbury, CT, USA), then transferred to regeneration induction medium (RIM). The experiment was made in a completely randomized design in trifactorial scheme 3 x 4 x 2, being three caps types, four sonication times, and two explant type; all with six replications per treatment. Each replicate consists by 10 explants placed in a 90 x 15 mm polystyrene Petri dishes containing 25 mL of RIM. After sonication, the explants remained on RIM consisting of MS salts, Nitsch vitamins (Nitsch, 1969), 0.1 g L^{-1} myo-inositol (Sigma-Aldrich Chemical Co, part number G1914), 2.0 mg L^{-1} zeatin (Sigma-Aldrich Chemical Co, part number Z0164), 0.01 mg L^{-1} indole-3-acetic acid (Sigma-Aldrich Chemical Co, part number I5148), 20 g L^{-1} sucrose, 6.5 g L^{-1} agar Merck (Merck KGaA, part number 232-658-1) at pH 5.8 ± 0.1, in a growth chamber with photoperiod of 16 hours, at 26 ± 2°C and 37 µmol m^2 s^{-1} irradiance.

After nine weeks of cultivation, the parameters were evaluated: living explants, total fresh weight (mg), fresh weight of living explants (mg), number of shoots higher than 0.5 cm and number of leaves. The ratios number of branches/explants, number of leaflets/explants, fresh weight/live explants and survival were assessed and calculated.

2.3. GAS EXCHANGE EVALUATION

The gas exchange flux measured in flasks covered with polypropylene caps PP, 1M and 2M MilliSeal^{TM} was done as previously described by Fujiwara and Kozai (1995). The headspace of each flask with different sealing type was filled with air saturated with carbon dioxide (CO_2) in a concentration of 25 mmol mol^{-1}. The inner concentrations of CO_2 were measured with a Headspace Gas Analyzer 6600 (IIlinois Instruments, Johnsburg, IL, USA). The amount of gas exchange per hour (N') in each sealing condition was estimated by the following equation: $N' = \frac{1}{T} \ln \frac{C_1 - C_{out}}{C_2 - C_{out}}$ (Fujiwara and Kozai, 1995), where T denotes the time (in hours) between two readings 1 and 2; C_1 and C_2 denotes to the inner CO_2 concentrations in the flasks at times 1 and 2; C_{out} is the CO_2 concentration in the environment external to the flask.

2.4. STATISTICAL ANALYSIS

The experiments were performed in a randomized design. Data were subjected to analysis of variance (ANOVA) and means were compared by test of Tukey ($P < 0,05$). All analyzes were performed using the statistical software Genes (Cruz, 2013).

3 RESULTS

3.1. INCREASE IN GAS EXCHANGE IMPROVED MORPHOLOGICAL STRUCTURES IN TOMATO GENOTYPES

For the cultivars Moneymaker and Santa Clara higher, gas exchange levels enabled by the membranes led to increased cotyledon area (Fig. 1A). The NCEBR-2 cultivar showed no difference in the cotyledon area among sealing types, however showed contrasting response in hypocotyl length (Figure 1B). To the hypocotyl length differences were observed among the sealing types within the same genotype.

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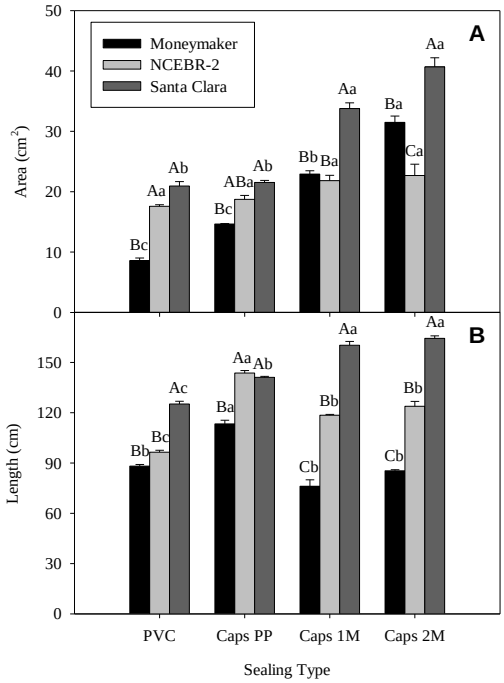

Figure 1. Cotyledon area (A) and hypocotyl length (B) of tomato genotypes (Moneymarker, NCEBR-2 and Santa Clara) under different sealing types [Plastic polyvinyl chloride (PVC) lids, Polypropylene lids (Caps PP), Lids with one membrane (Caps 1M), and Lids with two membranes (Caps 2M)], after 15 days in germination medium. Means indicated by the same capital letters did not differ among the sealing types and means indicated by the same lowercase letters did not differ among the genotypes by test of Tukey ($P < 0.05$).

Note with standing, growth and plantlet vigor were enhanced as higher the gas exchange was allowed by the use of membranes (Fig. 2). The shoots showed well-developed leaf primordia with normal characteristics in the treatments with the membranes. Conversely, in the treatments without membranes it was possible to visualize malformed leaves and hyperhydricity (Fig. 2A).

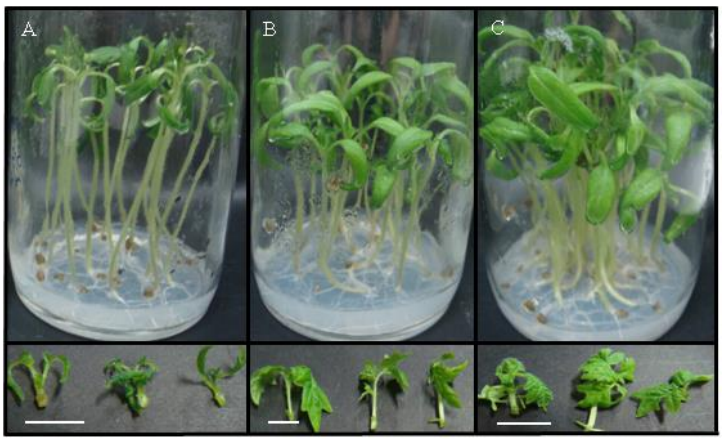

Figure 2. Development of 'Santa Clara' tomato seedlings under different sealing types, after 15 days in germination medium. (A) Polypropylene lids- Caps PP. (B) Lids with one membrane - Caps 1M and (C) Lids with two membranes – Caps 2M. Bar = 0.2 cm.

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3.2. THE ULTRASOUND APPLICATION ENHANCED ORGANOGENESIS

The sonication time influenced the following variables: number of shoots higher than 0.5 cm and number of leaflets. In addition to influences all the variables studied, there was a significative interaction between sonication and sealing type and between sonication and type of explant, showing the effect of exposure time to sonication in the morphogenesis. Regarding the total number of shoots, the highest results were achieved when three seconds of sonication and lids with two membranes were used. In the flasks with Caps 1M or Caps PP no increase with the use of sonication was observed. In the six and nine seconds treatments there were reductions in the number of shoots, regardless the sealing type (Fig. 3A).

In the leaflets formation, the three factors showed influence, and an interaction between sealing type and the sonication exposure time was observed (Fig. 3B), as well as between sealing type and type of explant (Fig. 4). For all sealing types used, hypocotyl was higher to cotyledons in leaflets formation (Fig. 4). For all the sonication exposure times tested, the higher number of leaflets occurred when Caps 2M or Caps 1M were used (Fig. 3B).

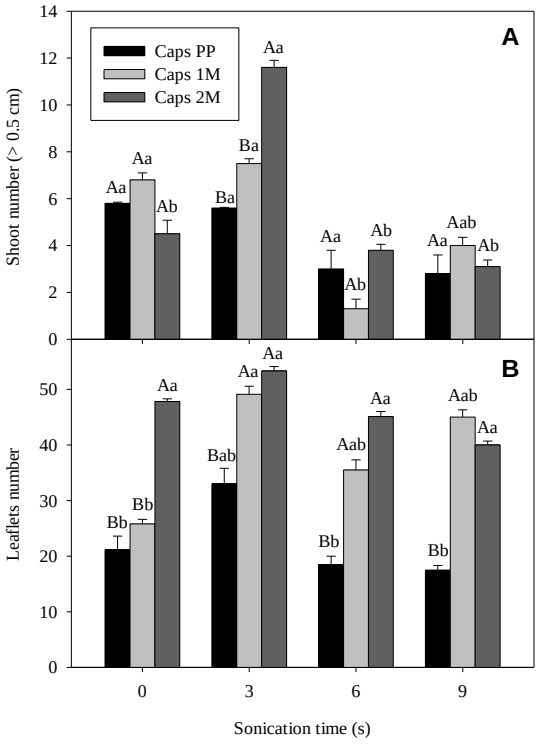

Figure 3. Shoot number (A) and leaflet number (B) in 'Santa Clara' tomato explants in response to different sealing types [Polypropylene lids without membranes (PP), Rigid polypropylene lid with 1M (Caps 1M), and Rigid polypropylene lid with two membranes(Caps 2M) and sonication exposure times (0, 3, 6, and 9 seconds), after nine weeks in regeneration induction medium. Means indicated by the same capital and small letters do not differ among sealing type and sonication time, respectively, by test of Tukey ($P < 0.05$).

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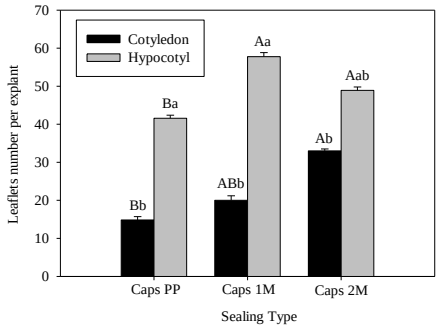

Figure 4. Number of leaflets in 'Santa Clara' tomato explants in response to different sealing types [Polypropylene lids without membranes (Caps PP), Polypropylene lids with one membrane (Caps 1M), and Polypropylene lids with two membranes (Caps 2M) and explant source (cotyledon and hypocotyl), after nine weeks in regeneration induction medium. Means indicated by the same capital and small letters do not differ among sealing type and explant source, respectively, by test of Tukey ($P < 0.05$).

The data analysis showed significant effect of sonication exposure time as well as its interaction with the explant source. When cotyledon explants were used, the highest number of shoots was achieved with three seconds of sonication, with a reduced response with longer times. The hypocotyl did not vary among different sonication exposure times (Fig. 5). In fact, the enhancement of organogenesis was evident in the interaction between higher levels of gas exchange and the ultrasound application (Fig. 6). The organogenesis was visually stimulated by the explant origin, presenting minor increase. However, when the explant morphogenesis has best provided by *in vitro* culture with facilitation of gas exchange, the application of ultrasound induces intensely responsive explants, resulting in high regeneration rates.

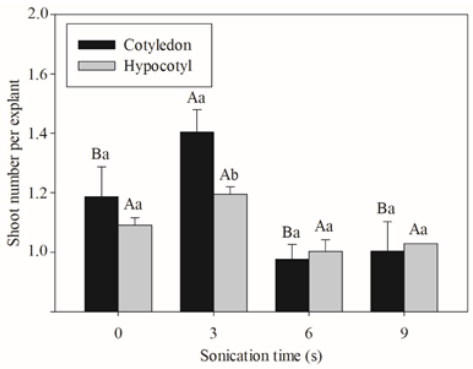

Figure 5. Shoot number in 'Santa Clara' tomato explants in response to different explant sources (cotyledons and hypocotyl) and sonication exposure times (0, 3, 6 and 9 seconds), after nine weeks in regeneration induction medium. Means indicated by the same capital and small letters do not differ among the sealing types and sonication exposure times, respectively, by test of Tukey ($P < 0.05$).

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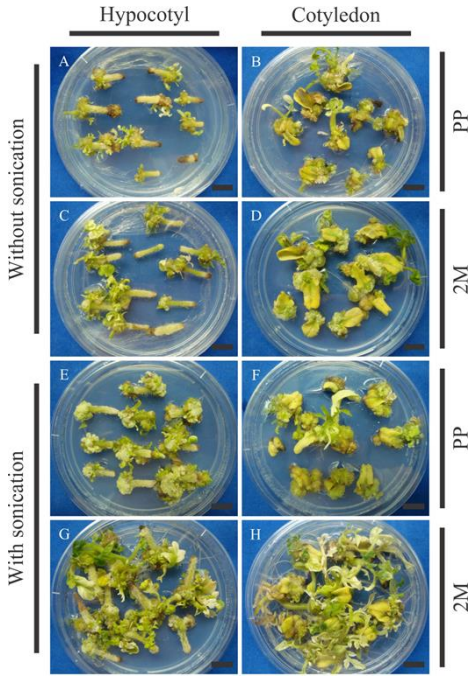

Figure 6. Organogenesis of 'Santa Clara' tomato explants in hypocotyl and cotyledons generated with and without enabling gas exchange membranes *in vitro* cultivation and application of ultrasound. After nine weeks of culture on regeneration induction medium, hypocotyls **A**, **C**, **E**, and **G** and cotyledons **B**, **D**, **F** and, **H** were treated as follow: **A**, and **B** - explants without exposure to sonication and without gas exchange**;** **C**, and **D** - explants without sonication and gas exchanges**;** **E**, and **F** - explants with and without sonication and gas exchange; **G** and **H** - explants with sonication and gas exchange. Bar = 1 cm.

4 DISCUSSION

In this study, we report for first time the regeneration increase by influence of interaction of physic factors: type of sealing and sonication. We demonstrate that higher gas exchange associated with sonication increased the *in vitro* regeneration in explants of model plants. The growth and morphological characteristics of tomato explants were increase by increased *in vitro* gas exchange. In general, closure systems that allow lower gas exchange *in vitro* cause low rates of transpiration and photosynthesis, which difficult the absorption of water and nutrients, and high rate of respiration in dark that reduces the explant growth rate (Kozai et al., 1997). Modifications of the microenvironment into the culture flask such as the enhance of gas exchange promoted by maintaining the CO_2 concentration appropriate lead to stimulate photosynthesis and reduce the ethylene concentration and the relative humidity inside the flasks (Kubota *et al.*, 2001), promoting growth *in vitro*, as observed in the present study.

The morphogenesis of cotyledon and hypocotyl explants of tomato was increased due to the condition inside the flasks, favored by the porous membranes, that allow for higher levels of gas exchange. The head space in the *in vitro* culture proved to be an important factor for morphogenic responses *in vitro*. Among the approaches that enable a better physiological condition, increasing gas exchange *in vitro* is an action that results in quantitative gains and reduces time to obtaining ideal

explants quantitatively and qualitatively (Kozai, 2010). In the present study, seedlings in flasks sealed with lids with Caps 2M had higher vigor, growth and visual quality. Flasks that allow higher gas exchange make *in vitro* inner conditions more similar to *ex vitro*, inducing changes of anatomical features in micropropagated shoots and leaves, which approximate the phenotype of *in vitro* plants to the *ex vitro* ones (Kozai, 2010). However, tomato seedlings grown under conditions of reduced gas exchange were more prone to hyperhydricity, whereas in conditions with increased gas exchange seedlings were possible more lignified and more resistant to water loss (Lai *et al.*, 2005; van der Dries *et al.*, 2013) In addition, in cultures with higher gas exchange, Mills *et al.* (2004) observed that deposition of cuticle on the leaf surface was higher, combined with a more developed vascular system and with increases in leaf area and dry weight.

In this study we found variations in the response of hypocotyl length among different sealing types. The genotype Moneymaker showed length reduction for increased gas exchange, while 'NCEBR-2' had variant levels of length in the same conditions and 'Santa Clara' showed growth increasing with higher gas exchange. The decrease in hypocotyl elongation in Moneymaker is corroborated by studies of Mohamed and Alsadon (2010), who assessed potato plantlets grown under different levels of gas exchange. It was found that shorter internodes and more leaves in ventilated flasks, with higher plants in non-ventilated flasks, possibly due the accumulation of ethylene. A good example of the morphological and physiological disorders that we often observe in conventional micropropagation but not in photoautotrophic micropropagation is hyperhydricity (vitrification). Hyperhydricity is reportedly caused by physical and chemical factors including high relative humidity and ethylene concentration inside the vessels, conditions, which do not exist in a photoautotrophic system (Santana-Buzzy *et al.*, 2006; Smith, 2013; Yadav *et al.*, 2003). Excess ethylene in the *in vitro* environment can inhibit the seedling establishment, besides being a main responsible to the induction of hyperhydricity, characteristic observed in shoot tips of seedlings obtained in sealed flasks containing explants of *Dianthus caryophyllus* (Yadav et al., 2003). The concentration of ethylene and morphological characteristics were studied in *Capsicum,* where chlorosis was observed in leaves and abscission of developing leaf primordia, along with rapid loss of force in unvented bottles with high concentration of ethylene (Santana-Buzzy et al., 2006).

The improvement in the expansion and vigor in seedlings caused by the increase of gas exchange occurred in all genotypes studied, especially for 'Moneymaker' and 'Santa Clara'. The knowledge of the morphogenic responses in each genotype is essential to achieve satisfactory protocols with high regeneration rates. Different tomato cultivars may have different physicochemical characteristics, organoleptic and physiological, such as sensitivity and response to ethylene and its modulators; the latter are directly related to the composition genotypic and allelic

interactions in each cultivar (Tigist *et al.*, 2013; Trujillo-Moya and Gisbert, 2012). Besides the influence of genotypic variability in regeneration process, several studies have verified the influence of different sources of explants, as well as the interaction of these two factors in *in vitro* morphogenesis of the genus *Lycopersicum* (Mercado *et al.*, 2000; Xu *et al.*, 2010; Yasmeen, 2009).

In this study, we verified the influence of gas exchange in the induction of organogenesis. The explants cultured *in vitro* under natural ventilation showed an increase in organogenesis by means of emission leaflets and branches. The use of the appropriate time of ultrasound favored regeneration in tomato explants in the environment with higher gas exchange. It is possible that sonication allows an increasing flux of hormones and nutrients to inside the plant cell or apoplastic medium that favored the intensity of organogenic responses in explants (Krikorian, 1995). The structure and the health of explants are factors that assists in organogenesis and genetic transformation, since the intense manipulation favors the occurrence of injuries that may cause the explants death. One of the immediate results from the high-intensity ultrasound application is the cell disruption (Santarém *et al.*, 1998). The surface damage allows then try of water, nutrients and growth substances to the explant, which can lead to a massive explant expansion and shoot regeneration (Ananthakrishnan et al., 2007).

In this study, the exposure of tomato explants to high sonication times caused a reduction in the number of shoots in cotyledons and hypocotyls. Likewise, Santarém et al. (1998) reported that sonication times superior to 5 seconds decreased or disabled soybean explants for competence to induce somatic embryos formation this was diminished or eliminated. Similarly, Ananthakrishnan et al. (2007) report that in higher ultrasound times, the explants were hyperhydrated, which was consistent with further surface ablation, uncontrolled water entry and also reduced the regeneration of squash explants. Nevertheless, longer treatments with ultrasound can be lethal to the explants (Meurer *et al.*, 1988; Santarém et al., 1998), and SAAT always reduced shoot proliferation in some soybean cultivars (Meurer *et al.*, 1998). In biological systems, the chemical effects of ultrasound involve the formation of free radicals that are formed in the final stages of transient cavitation, these free radicals can have an effect on the ability of splitting the remaining intact cells by affecting the integrity of membranes (Fu *et al.*, 1980). However, is notably difficult to compare ultrasonic treatments by different authors. There is a wide range of instrument types used with different geometries and nominal power outputs, and additionally a wide range of ultrasound frequencies are used (Liu *et al.*, 2006). Different instruments producing a wide range of frequencies and output power can produce similar results in SAAT. Ultrasound can be produced by other means *e.g.* powerful tunable instruments emitting via “horns” commonly used for tissue disruption, or by laboratory-produced equipment [(Liu et al., 2006) and references therein].

5 CONCLUSION

In this study, we showed for the first time the stimulation of organogenesis by the interaction of physical factors in *in vitro* cultivation. The use of good quality explants, obtained by *in vitro* culture with enhanced gas exchange and the application of ultrasound. We suggest the application of these factors as a significant new procedure to increase the quantity and quality of organogenesis and reducing the time consumed in the *in vitro* process, using simple and cheap treatments as facilitators caps gas exchange and ultrasound.

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CONFLICT OF INTEREST

All authors contributed substantially and approved the final submission.
All authors declare that they have no conflict of interest.

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