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Atmosphere modification system for respiration analysis of recalcitrant seeds during storage

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ABSTRACT: The modification of atmosphere composition is a strategy to prolong the storage of organisms susceptible to senescence. Atmospheres with high levels of carbon dioxide could be applied to prolong the viability of recalcitrant seeds, which are in a constant process of deterioration due to intense metabolic activity. However, there is a need to develop methods that allow the standardized generation of these modified atmospheres to isolate the effect of low oxygen from high carbon dioxide. In this study, the generation of modified atmospheres was carried out by experiments with insufflated gas (IG) or compensated vacuum (CV), on recalcitrant *Inga vera* embryos as an experimental model. In IG, CO_2 and N_2 were uninterruptedly injected into a mixing chamber, in which the gases underwent turbulence to be homogenized and subsequently applied to the flasks where the embryos were incubated. In CV, the embryos were placed in flasks, from which a fraction of the air present inside was removed, and subsequently, the gases of interest were injected in defined fractions. The CV method proved to be more efficient and it was possible to restrict the respiratory metabolism of the *Inga vera* embryos by reducing respiration with the increase in carbon dioxide applied exogenously.

Index terms: conservation, *Inga vera*, metabolic control.

RESUMO: A modificação da composição atmosférica é uma estratégia para prolongar o armazenamento de organismos suscetíveis à senescência. Atmosferas com elevados índices de dióxido de carbono podem ser aplicadas para prolongar a viabilidade de sementes recalcitrantes, que estão em constante processo de deterioração pela intensa atividade metabólica. Contudo, há necessidade de desenvolvimento de métodos que possibilitem a geração padronizável dessas atmosferas modificadas de forma a isolar o efeito da concentração de oxigênio da alta concentração de dióxido de carbono. Neste trabalho, a geração de atmosferas modificadas foi conduzida em experimentos com gás insuflado (GI) ou com vácuo compensado (VC) sobre embriões recalcitrantes de *Inga vera* (ingá), como modelo experimental. Em GI, CO_2 e N_2 foram ininterruptamente injetados em uma câmara para mistura, na qual os gases sofreram turbulência para serem homogeneizados e posteriormente aplicados nos recipientes onde os embriões foram incubados. Em VC, os embriões foram transferidos para frascos, de onde foi retirada uma fração do ar presente no mesmo, e posteriormente injetados os gases de interesse em frações definidas. O método VC se mostrou mais eficiente e foi possível restringir o metabolismo respiratório dos embriões de *I. vera* pela redução da respiração com o aumento do uso do dióxido de carbono aplicado exogenamente.

Termos para indexação: conservação, *Inga vera*, controle do metabolismo.

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INTRODUCTION

Modified atmospheres (MAs) have great potential for the control of the respiratory metabolism of seeds and, consequently, for the expansion of their conservation during storage. Such control is especially important for the development of technologies that allow the storage of recalcitrant seeds (sensitive to desiccation) for long periods. As a result of their intense metabolism, these seeds are constantly undergoing physiological stresses and cannot be dried or frozen to be stored (Barbedo, 2018; Xu et al., 2020), as orthodox, desiccation-tolerant seeds are normally stored (Redden and Partington, 2019). In seeds of *Eugenia brasiliensis* Lam. (grumixama), for example, increases in the concentration of carbon dioxide in the air resulted in a decrease in respiratory activity (Cécel and Barbedo, 2021), which could contribute to the preservation of these seeds for longer periods than the current limits.

The conservation of recalcitrant seeds in prolonged storage allowing their inclusion in germplasm banks, remains one of the greatest challenges of seed physiology and technology (Wyse et al., 2018). As they cannot have their water content reduced to values close to or below 10%, recalcitrant seeds also cannot be stored at negative temperatures, such as those used in seed banks. These two processes, i.e., the almost complete reduction of water content and the maintenance at freezing temperatures, are the main methods of reducing metabolic rates in orthodox (desiccation-tolerant) seeds, which allows their inclusion in germplasm banks for decades or centuries (Liu et al., 2018; Solber et al., 2020). Since it is not possible to adopt these methods for recalcitrant seeds, other forms of metabolism containment should be investigated, such as modifying the concentration of gases in the environment, i.e., the use of MAs.

MAs consist of techniques applied to spaces, packaging, or containers in which aspects such as gas balance are altered to extend the shelf life and viability of various foods. The main gases used in the generation of modified atmospheres are nitrogen, carbon dioxide and oxygen, aggregated in different proportions to meet the different objectives or character of the material to be conserved (Philips, 1996). In order to preserve different types of food, ranging from vegetables to meat and dairy products, MAs can often be used in association with other techniques, such as refrigeration, which, together, optimize conservation (Santos and Oliveira, 2012).

MAs are divided into active, passive, and vacuum. Active ones consist of the artificial and forcible alteration of the percentages of the gases of interest (Lana and Finger, 2000), mainly the reduction of oxygen supply. Lower availability of this gas tends to restrict respiratory activity and, therefore, the metabolism of the study materials, which reduces cellular activity and, consequently, the deterioration of fruits and vegetables (Arruda et al., 2011). Active MAs are divided into two systems: compensated vacuum and insufflated gas. The first consists of removing the atmospheric air present in the environment where the MA will be generated, leading to the formation of a vacuum. Subsequently, the desired gas mixture is injected into the environment and the vacuum is nullified. On the other hand, in the insufflated gas system, there is a constant injection of the desired gas mixture into the MA environment, so that the gas mixture attenuates the atmospheric gas parameters until the environment is completely filled by the gas mixture, which progressively replaces the previous atmosphere (Mantilla et al., 2010).

According to Weber et al. (2017), there are three main modern techniques for the evaluation of MAs: dynamic controlled atmosphere (based on chlorophyll fluorescence), dynamic controlled atmosphere based on ethanol (which evaluates the concentration of ethanol in the environment, which can be generated from alcoholic fermentation, indicating the low availability of oxygen), and dynamic controlled atmosphere based on respiratory quotient (which evaluates the relationship between the percentage of carbon dioxide produced and oxygen consumed, analyzing, among other things, which substrate was consumed during the generation of energy by the cells) (Weber et al., 2015).

MAs can become an important tool for increasing the conservation capacity of recalcitrant seeds, but there is a need to develop techniques for the production of MAs and to identify the participation of each modified gas in the biological processes altered in the seeds. In the study conducted by Cécel and Barbedo (2021), for example, the progressive insertion of carbon dioxide reduced the percentage of oxygen available to the seeds. Thus, it was not possible to attribute the reduction in respiratory activity specifically to the increase in carbon dioxide or to the

equivalent reduction in oxygen. Therefore, there is a need for methods that routinely enable the generation of MAs, with control of both CO_2 and O_2.

Thus, the objective of this study was to develop methods that allow the generation of MAs in a standardizable way, thus, allowing analyzing the reduction of respiratory metabolism of recalcitrant seeds. Subsequently, recalcitrant embryos of *Inga vera*, considered to be among the most recalcitrant known (Bonjovani and Barbedo, 2020; Pereira et al., 2020), were used as a model for controlling this respiration, seeking atmospheres that reduce the respiratory rates of these embryos during storage.

MATERIAL AND METHODS

Obtaining gases for the modified atmospheres

Two experiments were conducted to obtain the modified atmospheres (MAs), one with the insufflated gas (IG) system and the other with the compensated vacuum (CV) system. The gases used in the generation of MAs by IG were atmospheric air, nitrogen (N_2) and carbon dioxide (CO_2), while in MA by CV the gases used were N_2 and CO_2.

CO_2 was obtained from the reaction between sodium bicarbonate ($NaHCO_3$) and acetic acid (CH_2COOH). To this end, 100 g of sodium bicarbonate were placed in a 500 mL Kitasato flask. Next, the glassware opening was sealed with a rubber septum and plastic paraffin film. At the lateral outlet, a latex balloon was placed attached with string or elastic for the compensated vacuum system (Figures 1 and 2), or a cannula with a low-pressure valve that fed the inside of the flask called 'gas mixer' in the IG system (Figures 3 and 4). To ensure that the amount of air inside the system was minimal, atmospheric air was removed from inside the Kitasato flask, through the septum, using a 20 mL syringe and a needle, as much as possible, creating a partial vacuum inside the flask. After that, the same syringe was filled with a 20% acetic acid solution, which was injected into the flask through the septum, thus reacting with the sodium bicarbonate. To be on the safe side, the acid injection was done slowly, so that neither the generation of CO_2 nor the effervescence of the reaction would cause gas leaks. For the CV system, the acid injection stopped after filling the flask

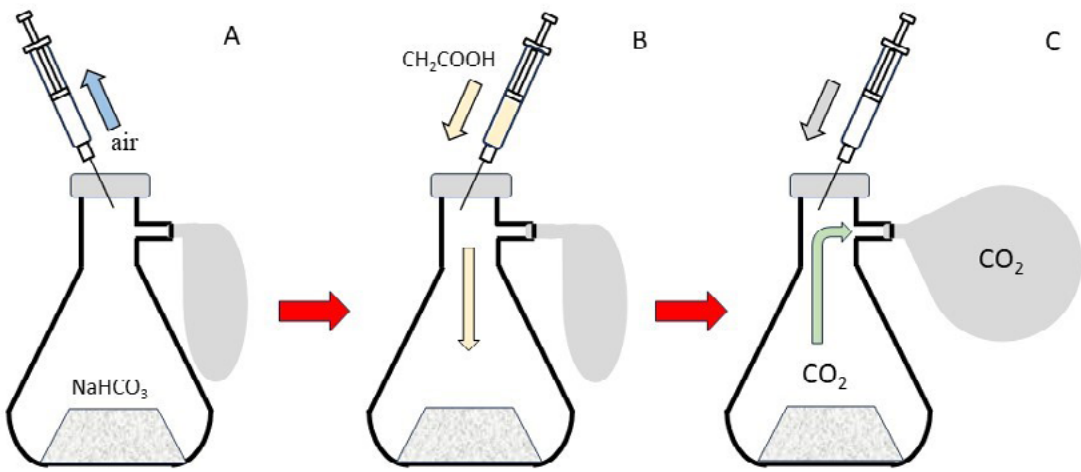

Figure 1. Schematic representation of the process of obtaining carbon dioxide. Initially, a syringe was introduced, through a rubber septum, into a sealed 500 mL Kitasato flask containing 100 g of sodium bicarbonate. Through this syringe, as much air as possible was removed from inside the Kitasato flask (A). Then, acetic acid (20%) was added to the Kitasato flask, using a syringe introduced through the septum (B). The reaction of sodium bicarbonate with acetic acid produced carbon dioxide, which, for the compensated vacuum system, was collected in a latex balloon attached with string or elastic to the lateral outlet of the Kitasato flask (C). After filling the balloon, the acid injection stopped.

with the gas. Subsequently, a low-pressure valve was placed in the opening of the balloon, which controlled the flow of the gas at the time of collection.

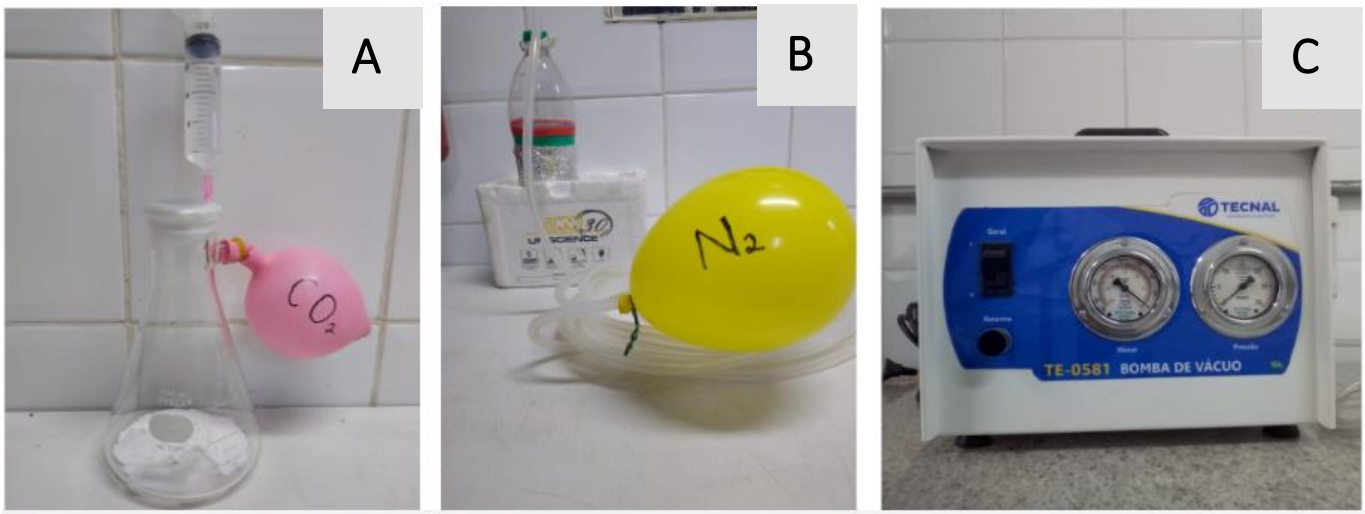

Figure 2. Equipment used in the production of gases. In A, Kitasato flask with sodium bicarbonate, syringe with acetic acid and latex balloon for CO_2 production and capture; in B, PET bottle with liquid nitrogen, in a polystyrene box, with a tube for conducting the gaseous N_2 to the latex balloon; in C, vacuum pump for atmospheric air capture.

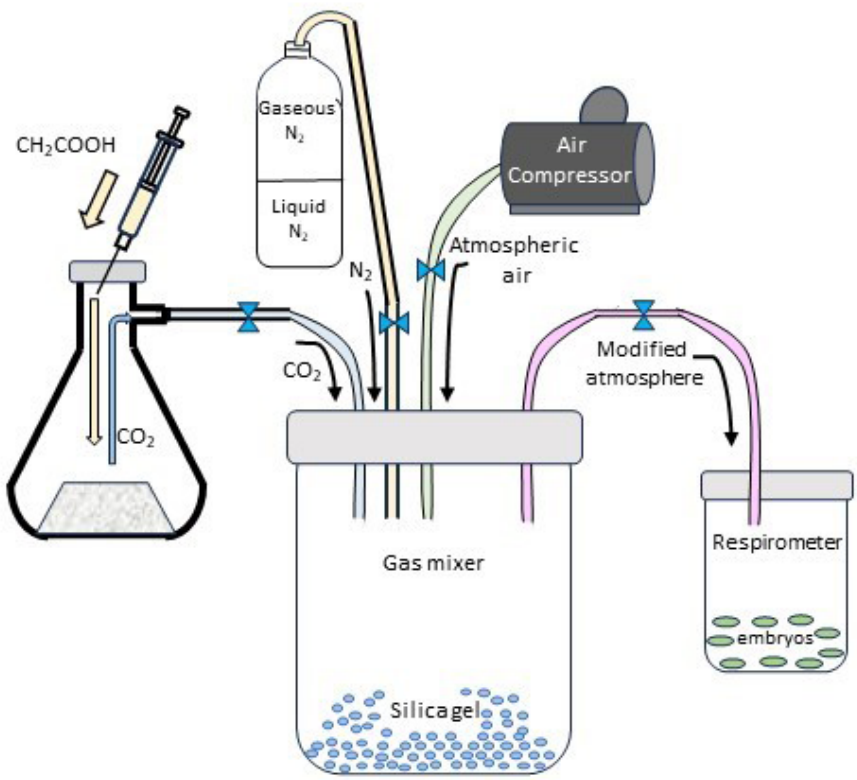

Figure 3. Schematic representation of the process of production of modified atmosphere by the insufflated gas system. CO_2, N_2 and atmospheric air are inflated into the gas mixer, controlling the access valves of each gas, until the desired gas mixture is obtained. From there, the access valve to the respirometer where the embryos to be studied are located is opened. For more details, see the description in Material and Methods.

N_2 was obtained through the evaporation of liquid nitrogen, obtained from a condenser, which liquefies N_2 from the atmosphere and filters it to values of approximately 99.99% purity, to ensure that contaminating substances, such as oxygen (O_2) and water, do not interfere with the processes in which N_2 is used. The liquefied N_2 was collected in a 2-liter PET bottle wrapped in aluminum foil and stored in a polystyrene box to control gas evaporation (Figure 2B). A long plastic hose was inserted into the bottle lid to ensure that the gas would reach room temperature as it evaporated. For the CV system, the gas was stored in latex balloons, with access only through a low-pressure valve attached by rubber elastic or string to the opening, through which the gas was accessed. For the IG system, the hose was connected directly to the gas mixer.

Atmospheric air, used only in the IG technique, was obtained from a Tecnal TE-0581 vacuum pump (*Tecnal Equipamentos Científicos*, Piracicaba), connected to a tube that was connected directly to the gas mixer (Figures 2C and 3).

The gases collected in balloons were used for the execution of the vacuum-compensated MA; for the insufflated gas technique, the N_2 outlet hose and the lateral outlet of the Kitasato flask, through which CO_2 exited after the reaction, were connected to the cannulas with low-pressure valves that fed the inside of the flask called 'gas mixer'.

Generation of MAs in the insufflated gas system

The chamber in which the MAs of the insufflated gas system were generated consisted of a transparent glass flask with a threaded opening, a plastic lid, and approximately 3 liters of volume, in which 500 mL of silica gel were placed, so that the gases would undergo turbulence and have the residual moisture removed (Figures 3 and 4A). In the lid of the flask, three holes were made for the entrance of cannulas that fed the inside of the flask with the gases; these holes were covered with a rubber septum for sealing and perforated in the central part for the passage of the cannula. In addition, a fourth hole with a rubber septum and cannula was made (Figure 4B) to move the gases to the final flasks, where the embryos were incubated for respiration evaluation, called 'respirometers'. These were characterized by 600 mL glass flasks with threaded opening, with a metal lid with a hole covered by a septum through which it was possible to insert the syringe needle (Figure 4D). The gas mixture was generated from the gases of interest in the mixer (Figure 3) and transferred to the interior of the respirometers via a needle cannula through the septum. CO_2 and O_2 concentrations were measured by collecting a sample of the air from inside the respirometer and injecting it into a 6600 gas meter (Illinois Instruments, Inc., Johnsburg, Figure 4E). In the respirometers, in addition to the needle that injected the gas mixture, a second needle was positioned in the lid, through the septum, so that the atmospheric air previously present inside the flask was removed by the inlet of the gas mixture, without generating an increase in the internal pressure of the system.

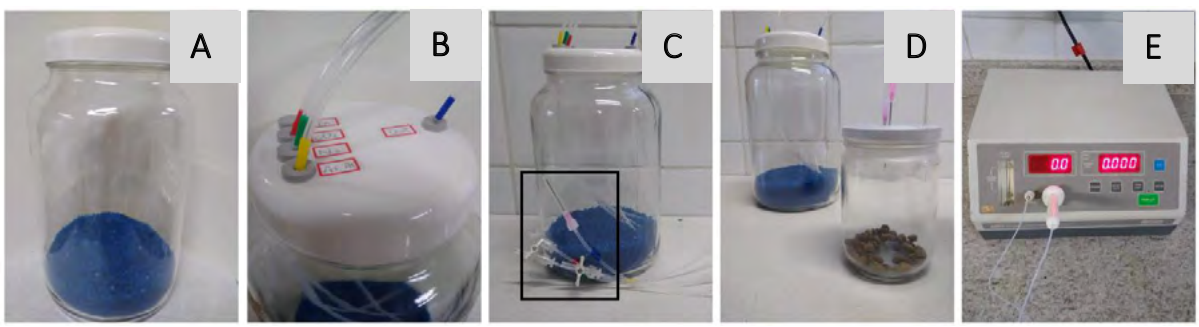

Figure 4. Equipment used to obtain the modified atmosphere by the insufflated gas system. In A, a glass bottle with a plastic lid containing silica gel, called a mixer; in B, lid with septa for the insertion of the gas cannulas on the left and the outlet of the gas mixture on the right; in C, complete mixer with detail of the gas inlet control valves and mixture outlet needle in the rectangle; in D, mixer outlet needle inserted into the respirometer for insertion under low pressure and extra needle for atmospheric air outlet and pressure maintenance; in E, Illinois 6600 gas analyzer used to check the percentage of gases.

Generation of MAs in the compensated vacuum system

In this system, the MAs were generated directly inside the respirometers, from the replacement of part of the atmospheric air present inside the flasks with gases of interest (in this case, N_2 and CO_2). The change in gas balance began with the reduction of O_2 concentration through the removal of part of the atmospheric air from the respirometer (Figure 5A), causing the amount of O_2 to be proportionally removed and replaced. To this end, based on preliminary results of gas changes from the insertion and removal of gases from the flasks, the following equation was developed: $V_{atm} = [(a – b) / (a . 10^{-2})] . c . 10$, *where* V_{atm} is the volume of atmospheric air to be removed from each respirometer to achieve the desired percentage value of O_2 (in mL), *a* is the O_2 concentration in the atmosphere, *b* is the O_2 concentration intended to be achieved, and *c* is the volume of the respirometer (in L). Thus, the volume of air to be withdrawn from a 600 mL flask to reach an 18% oxygen concentration was 83.2536 mL; this volume was later completed with the gases of interest, as described below.

To increase the CO_2 concentration, the following equation was developed: $V_{apl} = d . c . 10$, where V_{apl} is the volume of CO_2 to be applied to the flask to reach the desired value, *d* is the desired concentration of CO_2 and *c* is the volume of the respirometer (in L). Thus, after the removal of the calculated atmospheric air volume, 18 mL of CO_2 were injected into the respirometer (600 mL), to reach the modified atmosphere with 3% CO_2. Knowing this, the volume of CO_2 was subtracted from the value of atmospheric air taken from the flask (83 - 18 = 65 mL), and the remaining value was the volume of N_2 that was injected into the respirometer to reach the initial pressure again, inside the flask, before the removal of atmospheric air, thus avoiding the generation of partial vacuums. In the case of the generation of MA in 600 mL flasks without the addition of CO_2, the integral volume of 83.2536 mL was filled by N_2.

To remove or introduce air and gases into the respirometers, as soon as they were closed, a syringe attached to a cannula was inserted into the septum of the lid. The other end of the cannula had a small low-pressure valve, where the syringe tip was connected, to remove or inject the gases. Gas inflows and outflows were made through this cannula since the needle was already inserted in the lid of the respirometer. The purpose of this access with needle, cannula

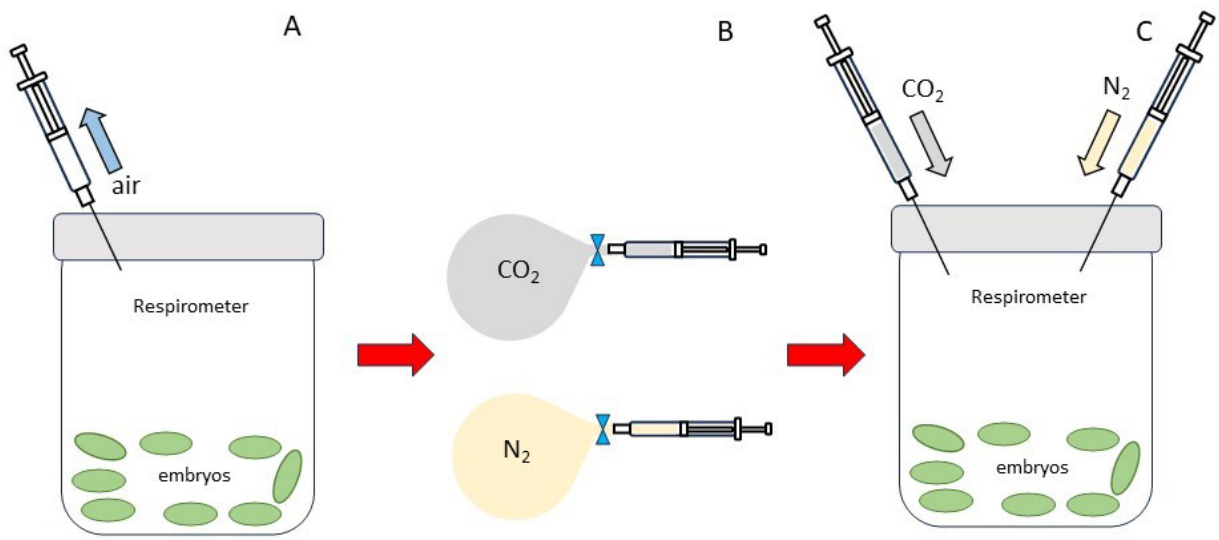

Figure 5. Schematic representation of the process of production of modified atmosphere by the compensated vacuum system. In A, the removal of air from the respirometer up to the desired O_2 levels; in B, collection of predetermined volumes of CO_2 and N_2 in syringes; in C, insertion of the gases in the respirometer to compose the modified atmosphere. Removal of air and insertion of CO_2 and N_2 were performed using the same syringe needle coupled to a cannula with a valve (not represented in this diagram). For more details, see the description in Material and Methods.

and valve was to avoid consecutive perforations in the septum of the flasks, which could increase injuries and gas leaks. As soon as the MA was generated inside the respirometers, O_2 and CO_2 values were checked on the Illinois 6600 gas analyzer (Figure 6), to ensure that the concentrations were equal to those desired for the experiment.

Incubation of embryos in MAs

Inga vera Willd. (DC.) T.D. Pennington embryos were obtained at Villa-Lobos Park (23°32' S; 46°43' W), São Paulo, SP, Brazil. Fruits were harvested manually when ripe, as described by Bonjovani and Barbedo (2020) and then taken to the Seed Analysis Laboratory, where their diaspores (embryos and sarcotesta) were extracted and washed. The sarcotesta was removed and the embryos were then washed under running water to remove any residues of sugars, which could compromise their storage. The embryos had the excess water from the washes removed with filter paper, and then they were stored in polypropylene plastic bags, where holes were made with a needle so that the material could breathe (Bonjovani and Barbedo, 2020).

I. vera embryos were incubated in 3 gas mixtures and under two temperatures. For the gas mixtures, one of them was considered control, i.e., with O_2 and CO_2 concentrations equal to those of the atmosphere (20.9% and 0.03%, respectively); the second gas mixture aimed to isolate the action of O_2, reducing its concentration, without adding CO_2 (18% of O_2 and 0% of CO_2), with the injection of N_2 as an inert gas to complete the volume of the flask; the last mixture of gases also maintained the O_2 level at 18%, but CO_2 was injected in this treatment, aiming at a concentration of 3%.

To evaluate the respiration of the embryos, four flasks (respirometers) with 15 *I. vera* embryos were used for each treatment. All respirometers were incubated at 5 °C for 5 days and at 25 °C for 1 day, based on previous studies to evaluate the respiration of these embryos. These periods were chosen based on the minimum time necessary for a sufficient change in the concentration of gases to occur, resulting from the respiration of the embryos, but before this alteration caused any damage to these embryos (Bonjovani and Barbedo, 2019, 2020; Parisi et al., 2019; Lamarca and Barbedo, 2021). At each temperature, the O_2 and CO_2 concentrations mentioned above were used. After the defined periods, O_2 consumption and CO_2 production by the embryos were evaluated in the respirometers, as previously described, calculating the respiratory rate and the respiratory quotient, as described by Kader and Saltveit (2002). At the end of the incubation periods, the embryos were removed from the respirometers and evaluated for viability by the germination test, as described in Bonjovani and Barbedo (2020). All experiments were conducted in a completely randomized design and the data obtained were statistically analyzed by the F test at 5% probability level. When significant, the means were compared with each other using Tukey test, also at 5% probability level (Santana and Ranal, 2004).

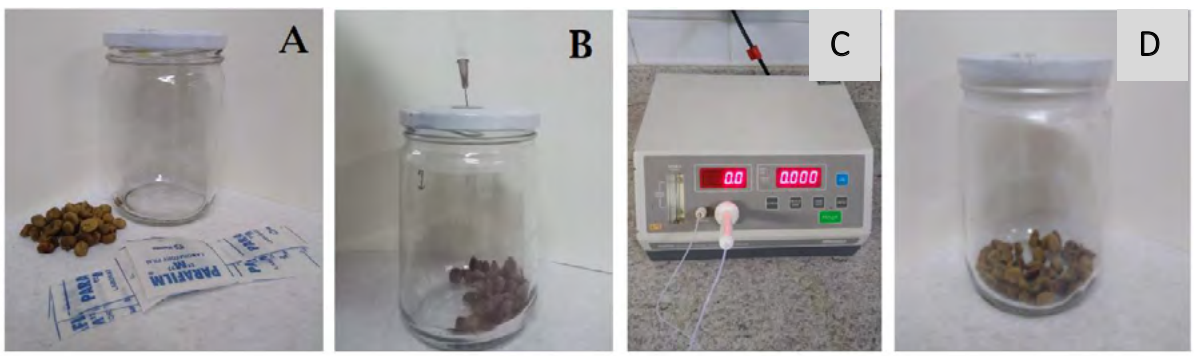

Figure 6. Equipment used to obtain modified atmosphere by the compensated vacuum system. In A, glass bottle with metal lid (respirometer), seeds and plastic paraffin film; in B, a respirometer with seeds and the needle that collects a sample to measure gases; in C, Illinois 6600 gas analyzer, which shows the percentages of oxygen and carbon dioxide; in D, respirometer already sealed by plastic paraffin film, before being taken to incubation.

RESULTS AND DISCUSSION

The generation of MAs from the compensated vacuum system proved to be more efficient since the desired values were already obtained in the second attempt (Table 1). On the other hand, in the insufflated gas system, even after 3 attempts, the O_2 concentrations were far below the desired level (Table 1). The mixture of gases in the insufflated gas system was exhaustive and imprecise, since it requires constant entry of gases under adequate flow to form the MA of interest, which requires precise control of nitrogen evaporation and the acid-base reaction to generate CO_2. In addition, the pressure generated by the flow of gases in the insufflated gas system was insufficient to perform the injection and replacement of gases in the respirometers. In the compensated vacuum system, the use of atmospheric air is dispensable, since the only gases used are N_2 and CO_2. In addition, as atmospheric air is replaced by specific gases, the balance control between gases is more balanced and precise.

Another important aspect to be noted is that the insufflated gas system required much larger volumes of gases to function properly, since it was necessary to adjust the gas flow rate and the efficiency of the mixer, as well as the pressure and dead volume, in this case. The compensated vacuum system, in addition to not requiring flow rate adjustments, also requires fewer gases and does not require the use of silica, since the seeds themselves can generate the turbulence necessary for the homogenization of the gas mixture.

Sealing by plastic paraffin film proved to be efficient, isolating the respirometers. However, it is advisable that the layer that covers the septum be maintained, in addition to the layer that covers the circumference of the lid-flask intersection region, as has already been done in previous studies evaluating the respiration of this same species (Bonjovani and Barbedo, 2019, 2020; Parisi et al., 2019; Lamarca and Barbedo, 2021) or even other species (Lamarca and Barbedo, 2012; Cécel and Barbedo, 2021). Sealing the gas mixer with plastic paraffin film could have reduced gas losses in the insufflated gas system. Despite requiring fewer gases, a smaller mixer would also have problems ensuring a constant flow of the gases of interest, since the speed of the reaction to generate CO_2 and the change in state of N_2 directly influence the pressure exerted on the insufflated gas system.

The equation for measuring the amount of atmospheric air that should be removed from the flask to reduce the percentage of oxygen proved to be efficient and applicable to other situations in which the objective is to reduce O_2 in other proportions. This is also true for the calculation of CO_2 enrichment. Combined, the two calculations can generate modified atmospheres of different standards, which meet the most diverse needs and objectives and, consequently, can restrict the final volume of N_2 in the mixture.

Obtaining of CO_2 and N_2 was efficient. However, removing a greater volume of air from the Kitasato flask at the time of CO_2 generation ensures greater purity of this gas. In addition, more concentrated acid solutions proved to be more efficient in reacting with sodium bicarbonate, although the risk of the system being overloaded by the pressure generated was greater.

Table 1. O_2 and CO_2 concentrations obtained after processes of production of modified atmosphere by the insufflated gas and compensated vacuum systems.

System	Attempt	Desired concentrations		Obtained concentrations	
		O_2	CO_2	O_2	CO_2
Compensated vacuum	1st	18%	3%	16%	4%
	2nd	18%	3%	18%	3%
	3rd	18%	0%	18%	0%
Insufflated gas	1st	18%	3%	2%	7%
	2nd	18%	3%	8%	5%
	3rd	18%	3%	9%	3%

Due to the inefficiency of the insufflated gas system in producing the desired MAs, the incubation of *I. vera* embryos in the different MAs was performed only with the compensated vacuum system.

The analysis of seed respiration can be an important tool for diagnosing seed metabolism (Gao et al., 2023). In general, the embryos had more intense respiratory activity at 25 °C than at 5 °C (Table 2), which was already expected since the intense metabolic activity of these embryos when the temperature is higher has already been demonstrated (Pereira et al., 2020), as observed in corn seeds (Valle et al., 2021), which are orthodox. This result can be explained by the reduction in metabolic activity as a consequence of low temperatures, which affects enzymatic action (Ruelland and Zachowski, 2010). Despite the reduction in metabolic activity with the decrease in temperature, it is important to note that even at negative temperatures respiration can still occur, as observed for *I. vera* embryos at -2 °C (Bonjovani and Barbedo, 2020). Comparatively, the embryos in the treatments under high temperatures, as observed in Figure 5, showed a higher respiratory rate, which indicates greater metabolic activity and may culminate more quickly in the deterioration of these diaspores, as also observed in *Caesalpinia echinata* (Lamarca and Barbedo, 2012). At a temperature of 5 °C, with initial concentrations of 3.0% CO_2 and 18.0% O_2, the values of these gases were the same as their concentrations in the normal atmosphere, suggesting gas leakage in the respirometers. This did not allow the inclusion of this treatment in the analysis of the other results.

At 25 °C, the reduction in O_2 concentration caused a reduction in O_2 consumption by the embryos, regardless of whether or not the CO_2 concentration was altered (Table 2), suggesting that the lower oxygen availability could have reduced respiratory rates. However, when analyzing the changes in the release of CO_2 by these embryos, it was observed that the lower consumption of O_2 may have been in fact the result of lower rates of oxidative processes other than respiration, since the CO_2 values did not change when only the concentration of O_2 in the respirometer was changed. This hypothesis is corroborated by the fact that the RQ (respiratory quotient) increased (Table 2). In fact, respiratory activity was reduced when there was an increase in the concentration of CO_2 in the respirometer (Table 2). The respiratory rate in the CO_2 enrichment treatment was the lowest of the three, as observed in *Eugenia brasiliensis* (Cécel and Barbedo, 2021). At 5 °C, there was no significant change in O_2 consumption or CO_2 release with the MAs. In this case, it is possible that the incubation time of the embryos was not sufficient to detect differences in respiratory activity.

The RQ in the treatments with 20.9% O_2 and 0.0% CO_2, at both temperatures and 18.0% O_2 and 3.0% CO_2, at 25 °C, was close to or lower than 0.5, which may indicate oxidative activity; in the other treatments, the RQ of 0.66 may indicate that the embryos were consuming lipids as a substrate for their respiratory process (Table 2). Additionally, RQ values greater than 1 may indicate that the embryos could be fermenting (Kader and Saltveit, 2002).

Table 2. O_2 consumption, CO_2 release and respiratory quotient (RQ) of Inga vera embryos incubated at two temperatures, two O_2 concentrations and two CO_2 concentrations.

Gas concentration, temperature (T) and embryo incubation time (t)				O_2 consumption by the embryos	CO_2 release by the embryos	RQ
$[O_2]$	$[CO_2]$	T	t	(µmol.gDM^{-1}.day^{-1})	(µmol.gDM^{-1}.day^{-1})	
20.9%	0.0%	25 °C	1 day	123.53 a*	60.18 a	0.48 c
20.9%	0.0%	5 °C	5 days	24.07 c	12.08 bc	0.50 c
18.0%	0.0%	25 °C	1 day	96.34 b	63.47 a	0.66 b
18.0%	0.0%	5 °C	5 days	6.02 c	10.21 c	1.20 a
18.0%	3.0%	25 °C	1 day	87.49 b	23.09 b	0.26 d
18.0%	3.0%	5 °C	5 days	-	-	-

*Means followed by the same letter in the columns do not differ from each other by Tukey test at 5% probability level.

Table 3. Germination of *Inga vera* embryos at the end of incubation treatments at different temperatures and different modified atmospheres.

Temperature	25 °C			5 °C		
O_2 concentration	20.9%	18.0%		20.9%	18.0%	
CO_2 concentration	0.0%	0.0%	3.0%	0.0%	0.0%	3.0%
Germination	96% a*	99% a	99% a	95% a	95% a	94% a

*Means followed by the same letter, in the rows, do not differ from each other by Tukey test at 5% probability level.

Most of the embryos remained alive during the incubations, as they reached values close to 100% germination in all treatments (Table 3). In respiration evaluations by the method of analysis of the concentration of gases in respirometers, the incubation time of the embryos should not allow them to lose viability, since the change in the respirating mass would lead to inaccuracies in the analysis of respiratory rates. On the other hand, the incubation time must be sufficient for the respiration of the embryos to cause changes in the concentration of gases in the respirometer. The increase in CO_2 concentration, or the reduction of O_2, resulting from the respiration of embryos (or seeds), can lead many to death (Cécel and Barbedo, 2021) in *Eugenia brasiliensis* seeds. As the *I. vera* embryos remained alive at the end of the incubations, the incubation time was correct for the temperature of 25 °C, but it may have been insufficient for the temperature of 5 °C. The change in the composition of the gases in respirometers depends, among other factors, on temperature, respirometer air volume, and respirating embryo/seed mass. As a result, several combinations between temperature and incubation time can be found in different studies with embryo/seed respiration (Lamarca and Barbedo, 2012; Parisi et al., 2019; Bonjovani and Barbedo, 2020; Lamarca and Barbedo, 2021).

By analyzing the respiration results of the present study, it was observed that the reduction of the respiratory rates of *I. vera* embryos occurred essentially when the temperature was reduced or when the concentration of CO_2 in the atmosphere of the respirometers was increased. The reduction in respiratory rates may indicate a reduction in the general metabolism of these embryos, providing important support for the development of techniques that allow the conservation of recalcitrant seeds for prolonged periods, since the intense metabolism of these seeds has been shown to be the main factor in their rapid deterioration (Barbedo, 2018). Prolongation of seed storage capacity with the increase in CO_2 concentration in the atmosphere was observed in *Triticum durum* seeds, which are tolerant to desiccation and have low water content (Bennici et al., 1984). In the present study, it was demonstrated that metabolic rates can also be reduced in seeds sensitive to desiccation and with high water content. Controlling the atmosphere for storage of these seeds can, therefore, help in the search for the technology for their storage, especially the controlled increase of CO_2.

CONCLUSIONS

The compensated vacuum system was the one that promoted the best results for obtaining a modified atmosphere for *I. vera* embryos. The atmosphere modified by the increase in CO_2 concentration made it possible to reduce the respiratory rates of *I. vera* embryos.

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