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Research

Sustainable Protocols For Leaf Sample Collection in *In-Vitro* Culture: Evaluating The Impact of Sample Bags and Hydrogen Peroxide Pre-Sterilization

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ABSTRACT

This study investigates sustainable leaf sample collection protocols for *in-vitro* culture (IVC) of chili (*Capsicum frutescens*) and tomato (*Solanum lycopersicum*) leaves. The research aimed to enhance viability and reduce contamination of leaf explants by evaluating various types of sample bags and different concentrations of hydrogen peroxide (H_2O_2) pre-sterilization. Specifically, the study compared the effectiveness of normal sealed (NS) bags and vacuum-sealed (VS) bags, including high-action (VSH) and low-action (VSL) vacuum-sealed bags, alongside H_2O_2 pre-sterilization at various concentrations. Leaf samples were treated with ten different concentrations of H_2O_2 (5% to 50%) to assess their impact on necrosis and surface contamination over 72 hr. Results showed that higher H_2O_2 concentrations (above 30%) caused significant necrosis, while concentrations between 10% and 15% provided optimal pre-sterilization for both leaf types, effectively reducing contamination without excessive tissue damage. In the second phase, the research examined the influence of different sample bags on leaf explant sustainability. Vacuum-sealed bags, particularly those with low-action vacuum (VSL), significantly improved leaf longevity and minimized contaminant emergence compared to normal sealed bags. Combined with 15% H_2O_2 pre-sterilization, VSL bags performed best, maintaining leaf morphology and viability for extended periods. Statistical analyses confirmed the significant impact of sampling bag type and pre-sterilization on contamination levels, necrosis emergence, and leaf longevity. The findings suggest that using low-action vacuum-sealed bags (VSL) with 15% H_2O_2 pre-sterilization is a promising approach for sustainable leaf sample collection, enhancing the success rate of IVC by minimizing microbial contamination and preserving leaf integrity during transport. This optimized protocol offers valuable insights for researchers and practitioners in plant tissue culture and agriculture, aiming to improve the sustainability and efficiency of leaf sample collection for *in-vitro* applications.

Key words: *Capsicum frutescens*, hydrogen peroxide (H_2O_2), *in-vitro* culture, leaf sample collection protocols, *Solanum lycopersicum*, vacuum-sealed bags

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INTRODUCTION

In-vitro culture (IVC) is a pivotal technique in plant tissue culture (Hussain *et al.,* 2012), essential for various applications including conservation of rare or endangered plant species (Kulak *et al.,* 2022; Rani *et al.,* 2023; Thakur, 2024), genetic modification (Setamam *et al.,* 2014; da Chuna *et al.,* 2021; Purwantoro *et al.,* 2022); disease resistance studies (Huang *et al.,* 2020); plant development (Wijerathna-Yapa & Hiti-Bandaralage, 2023); and many recent advancements in micropropagation methodologies (Norouzi *et al., 2022*; Hasnain *et al.,* 2022; Dogra, 2023). The IVC process involves placing small explants, such as leaves (Sakthivel & Manivannan, 2021), seeds (Junaidy & Shahruddin, 2021), internodes (Biasi *et al.,* 2000), meristems, or cotyledon (Shafiq *et al.,* 2022), in a sterile, nutrient-rich medium where they can proliferate (Hussain *et al.,* 2012). In the case of leaf explants from *Capsicum frutescens* and *Solanum lycopersicum*, these samples are extensively used in plant tissue culture for various biotechnological applications (Setamam & Sidik, 2017; Sakthivel & Manivannan, 2023), which offer significant advantages, including rapid cloning

and the ability to propagate elite cultivars efficiently, allowing for large-scale production of plants with desirable traits (Ahmed *et al.,* 2023; Umar *et al.,* 2023), especially in chilies (Lopez-Moreno *et al.,* 2023) and tomato (Molitor *et al.,* 2023) agriculture crops.

The success of IVC heavily depends on the quality and viability of the leaf explants used. Fresh leaf samples that maintain their structural integrity are necessary to ensure successful culture outcomes (Tisserat & Vandercook, 1985; Pereira *et al.,* 2000; Ramgareeb *et al.,* 2001), particularly when facing issues with long-distance transport and outfield sampling. However, traditional leaf sample collection methods, commonly designed for nutritional analysis, herbarium, and other biological chemistry-related studies (Ossola, 2024), which often require samples to be dried (Thandapani, 2024), fail to meet the unique needs of the critical role of freshness in tissue culture success (Stamp *et al.,* 1990). Less freshness in the leaf sample commonly causes microbial contamination and vulnerability to viral and fungal pathogens (Ha *et al.,* 2020), complicating the IVC process and often resulting in a failure outcome (Moreno-Vázquez *et al.,* 2014). A recent study showed that the high microbial diversity of both spoilage bacteria and fungi significantly lowered the impact of tomato IVC success (Gerszberg & Grzegorczyk-Karolak, 2019). Similarly, in the latest chili study, contamination and phenolic exudation that inhibit tissue growth were well documented during IVC (Rachmawati *et al*., 2023). Despite rigorous sterilization efforts, microbial contamination remains a significant challenge (Abdalla *et al.,* 2022) due to surface sterilization resistance, which often competes with plant tissues for nutrients and potentially causes severe economic losses due to reduced shoot proliferation and tissue necrosis (Cardoso *et al.,* 2018; Abdalla *et al.,* 2022; Silva *et al.,* 2022). This is especially problematic when contamination levels on explant surfaces (Hashim *et al.,* 2021) are high and become evident only after several subcultures (Hindoy *et al.,* 2020; Permadi *et al.,* 2023). Despite these challenges, leaf explants remain essential in plant biotechnology for rapid cloning and plant development due to their ease of access (Sanatombi & Sharma, 2008; Cruz-Mendívil *et al.,* 2011; Zaytseva, 2024).

To address these challenges, the oxidative properties of H_2O_2 provide a versatile tool for maintaining aseptic conditions in IVC setups, particularly for explant surface sterilization (Curvetto *et al*., 2006). H_2O_2 exhibits broad-spectrum antimicrobial (Linley *et al*., 2012) and insecticidal efficacy properties (Rafael *et al*., 2001), including against bacterial spores, viruses, and yeasts (Linley *et al*., 2012). In early studies, at low concentrations, H_2O_2 demonstrated germicidal and fungicidal activities without adversely affecting *in vitro* seed germination or plantlet growth (Rafael *et al*., 2001; Curvetto *et al*., 2006). Being a neutral and small-sized molecule (Hen *et al*., 2017), H_2O_2 rapidly penetrates microorganism membranes, triggering the production of free hydroxyl radicals and oxidation of DNA (Chihara *et al*., 2018), proteins, and membrane lipids without generating toxic by-products (Linley *et al*., 2012; Hen *et al*., 2017). The lack of cellular death, or necrosis, is attributed to plant peroxidases decomposing H_2O_2 into water and oxygen, thus providing a protection mechanism against toxicity effects (Anderson *et al*., 2024). This makes H_2O_2 particularly suitable for use in IVC, maintaining the delicate balance between disinfection and phytotoxicity (Yanagawa *et al*., 1995; Curvetto *et al*., 2006; Noszticzius *et al*., 2013).

Another challenge is that the preparation of fresh leaf explants involves cutting, which can cause phenolic exudation from explants (Hamdeni *et al*., 2022), attract microbes, and increase susceptibility to pathogens (Leifert & Cassells, 2001). This condition hastens metabolic rates during storage, resulting in necrosis (Spokowski, 2010). The breakage of internal plant cell compartments allows enzymes and substrates, such as polyphenol oxidase (PPO) and peroxidase (POD) (Wanakamol *et al*., 2022), to cause browning reactions and chlorophyll degradation (Rahayu *et al*., 2019). This promotes microbiological growth and spoilage, further reducing sample quality (Wanakamol *et al*., 2022). Conventional PVC-sealed bags for food packaging that are commonly used for sample collection fail to preserve the freshness and integrity (Jacobsson *et al*., 2003; Olarte *et al*., 2009) of leaf samples needed for IVC, resulting in high rates of contamination, necrosis, and overall poor viability that showed similar results in the study of vegetable commercial PVC storage (Akomolafe & Awe, 2017).

However, vacuum-sealed (VS) bags, which create anaerobic conditions by removing air before sealing, can inhibit the growth of aerobic bacteria and extend sample shelf life (Djordjevic, 2017). This technique, shown to maintain quality and extend shelf life by inhibiting oxidative reactions and microbial growth, helps slow down respiration (Spokowski, 2010) and ethylene production (Jacobsson *et al*., 2003), prolonging leaf sample shelf life (Gorris & Peppelenbos, 1992). Thus, the combination of the oxidative properties of H_2O_2 and vacuum-sealed bags can have significant potential to enhance the preservation of leaf integrity and viability by reducing the microbial load without causing damage to the plant tissues.

Therefore, this study aims to develop and evaluate sustainable leaf sample collection protocols specifically tailored for the IVC of *Capsicum frutescens* and *Solanum lycopersicum* leaves. The specific

objectives are to: compare the effectiveness of normal sealed (NS) bags and vacuum-sealed (VS) bags, including both high-action (VSH) and low-action (VSL) vacuum sealing, in maintaining leaf viability and reducing contamination; evaluate the impact of various concentrations of hydrogen peroxide (H_2O_2) pre-sterilization (ranging from 5% to 50%) on necrosis and surface contamination; and identify the optimal combination of bag type and pre-sterilization method to ensure maximum leaf longevity, minimal contaminant emergence, and preserved leaf morphology during long-distance sampling. Addressing this problem of inadequate leaf sample collection protocols for IVC is crucial for improving the success rate and efficiency of plant tissue culture applications, particularly for chili and tomatoes.

Continuous advancements in research and technology are making significant strides in overcoming these obstacles (Abdalla *et al*., 2022). The substantial benefits, including the rapid production of disease-free, genetically uniform plants, make tissue culture an invaluable tool in plant biotechnology and sustainable agriculture (Purwantoro *et al*., 2022). Developing an optimized protocol will provide a reliable, cost-effective, and time-efficient method for collecting leaf samples, minimizing microbial contamination, and preserving leaf integrity, especially during outfield sampling.

MATERIALS AND METHODS

Materials

In this experiment, *C. frutescens* var. bird eye chilies (Figure 1a) and *S. lycopersicum* var. cherry tomato (Figure 1b) from the Solanaceae family were the main subjects of interest. More than 30 to 40 healthy young plants were obtained from a specific local nursery for a constant high-quality plant. Any disease-potential plant was discarded immediately.

Methods

Leaf explant sample collection

The leaf explants from *C. frutescens* and *S. lycopersicum* had been observed thoroughly with macroscopic observation, with normal and abnormal leaves noted. A clean, neat, and good condition *C. frutescens* leaf samples exhibit a shiny waxy layer (adaxial), a smooth surface with reduced trichomes (both sides), dark green coloration with a curved tip, and smooth edges in lanceolate shape (Figure 1 (a & b). While green-colored *S. lycopersicum* leaves exhibit the presence of abundant trichomes throughout both sides of the leaf with serrated ovate shapes (Figures 1(c) & d) were collected as samples.

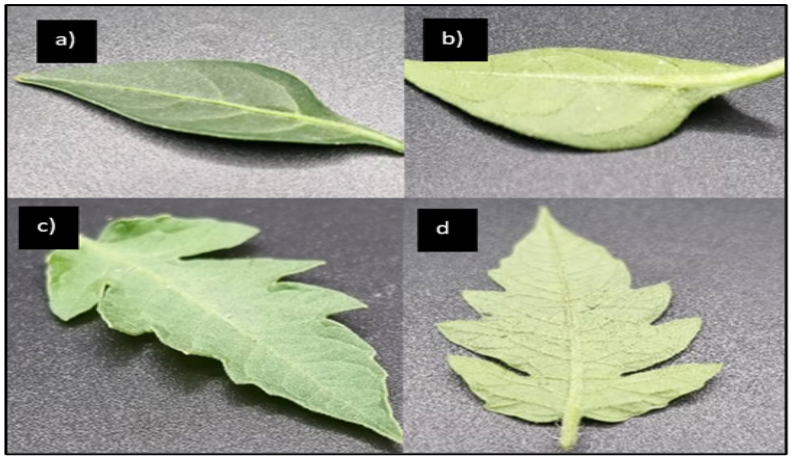

Fig. 1. Normal and good condition of young leaf sample suitable for IVC: a) adaxial *C. frutescens*; b) abaxial *C. frutescens*; c) adaxial *S. lycopersicum*; d) abaxial *S. lycopersicum*.

Hydrogen Peroxide for pre-sterilization of leaf explant surface

Around 30 leaf samples were selected, and pre-sterilization of the leaf surface by using H_2O_2 was done with the foliar spray technique in less than a min. Then, the leaves were quickly rinsed with distilled water and properly dried out with tissue paper before being stored in different types of sealed bags. The experiment was started with 50% of the highest concentration of H_2O_2 which was later followed by a 5% reduction treatment.

Testing the different types of sealed bags for leaf explant sample

Approximately ten leaves were collected in each sealed bag (*n*=10). In normal-sealed (NS) bags, the leaves were gently placed together during a short-duration opening and immediately sealed. For the vacuum-sealed bags, the leaves were arranged approximately 2–3 cm apart without overlapping before using a manual hand pump vacuum. The portable vacuum suction was applied in two ways: high-action vacuum (VSH) and low-action vacuum (VSL). The VSH was applied by completely pressing the plastic seal against the leaf surface, ensuring no room for air, gaps, or movement. In contrast, the VSL vacuum-sealed bags were applied by leaving a small gap or air around the leaves, allowing the plastic seal to show no contact but still restricting leaf movement within the bag. Both types of leaf sample collection bags were kept in a drying cabinet with 45% relative humidity at room temperature for two weeks. Observations were made for any changes in leaf necrosis and contaminant emergence.

Murashige & Skoog (MS) media preparation for in-vitro culture

MS media was prepared by adding 4.4 g of powder to 1.0 L of sterile distilled water. The pH was adjusted to approximately 5.7–6.0 using hydrochloric acid (HCl) or sodium hydroxide (NaOH). About 4.0 g of agar was added before autoclaving at 121°C and 15 psi for 20 min. The leaf samples were treated without or with optimal pre-sterilization at 15% H_2O_2 and further aged in each type of sampling bag for ≤ 24 hr and ≤ 72 hr. In addition, before the IVC began, all sampling bags containing potential good-condition leaf explants received similar treatment during storage, which was 45–65% RH at room temperature between 24°C and 26.5°C. The leaf explant samples from both *C. frutescens* and *S. lycopersicum* were cut to approximately 3–4 cm before being cultured in basal MS media for one month. All infected or necrotic explants were discarded before basic sterilization, in which the sterilization process involved treating the explants with 70% ethanol and 50% sodium hypochlorite with Tween 20, followed by rinsing with distilled water.

RESULTS AND DISCUSSION

The effect of different concentrations of pre-sterilization of hydrogen peroxide on leaf explant surface

The experiment was conducted using ten different concentrations of H_2O_2 (5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, & 50%), and the effects of necrosis on the leaf samples after applying each H_2O_2 concentration were recorded over 72 hr and presented in line graphs showing necrosis percentages (Figures 2 & 3). Over the three days of observation, necrosis showed a linear and proportional increase with higher H_2O_2 concentrations in both *C. frutescens* and *S. lycopersicum*. The results indicated that necrosis was slightly lower in *C. frutescens* compared to *S. lycopersicum* for all H_2O_2 treatments. This difference may be attributed to the presence of a wax cuticle layer in *C. frutescens*, providing a more effective liquid-proof shield compared to *S. lycopersicum* (Zeisler-Diehl *et al*., 2018). Even without H_2O_2 pre-sterilization (control), necrosis emerged with percentages similar to those observed with 5%–15% H_2O_2 treatments after 72 hr, showing 7.17% necrosis in *C. frutescens* and 12.7% necrosis in *S. lycopersicum* was mainly due to infection and trapped humidity (Akomolafe & Awe, 2017; Wanakamol *et al*., 2022) within the leaf samples.

Furthermore, the graphs in Figures 2 and 3 showed that within the first 24 hr, H_2O_2 concentrations ranging from 5% to 30% resulted in less than 10% necrosis emergence in both leaf types. However, concentrations above 35% H_2O_2 demonstrated potential hazards as an aggressive oxidizer (Gagnaire *et al*., 2002; Chihara *et al*., 2018), causing corrosion in living cells. This effect was particularly pronounced in *S. lycopersicum*, which showed higher necrosis emergence after 24 hr at these higher concentrations. Likewise, the sudden spikes in necrosis after 60 hr for 20%–30% concentrations made these less suitable for pre-sterilization of both *C. frutescens* and *S. lycopersicum* leaf samples. Meanwhile, lower concentrations of H_2O_2, specifically 5%, 10%, and 15% were able to maintain necrosis below 15% after 72 hr in both species. This slight necrosis was likely due to the instability of H_2O_2 bonds, which are easily broken, initiating reactive processes (Yoo, 2018).

It was noted that unattached leaves from the plant contain enzymatic catalases and peroxidases that act against excessive H_2O_2 by transforming it into water and oxygen (Curvetto *et al*., 2007), thereby preserving plant cells from necrosis effects regardless of internal H_2O_2 secretion or external sources (Rafael *et al*., 2001; Curvetto *et al*., 2007). However, once the plant was collected during sampling, these enzymes no longer provided protection, leading to necrosis when external H_2O_2 was introduced. Depending on the plant type, the explant sample might exhibit a delayed reaction to H_2O_2 and different necrosis effects due to the breakdown of H_2O_2 bonds (Aasim *et al*., 2013; Anderson *et al*., 2024). Therefore, for the durability of *C. frutescens* and *S. lycopersicum* leaf samples, the optimum pre-sterilization showed low levels of necrosis with no spike in browning, which indicated that a pre-

sterilization treatment of 15% H_2O_2 for one min was able to maintain leaf explant morphology with green coloration and minimal necrosis.

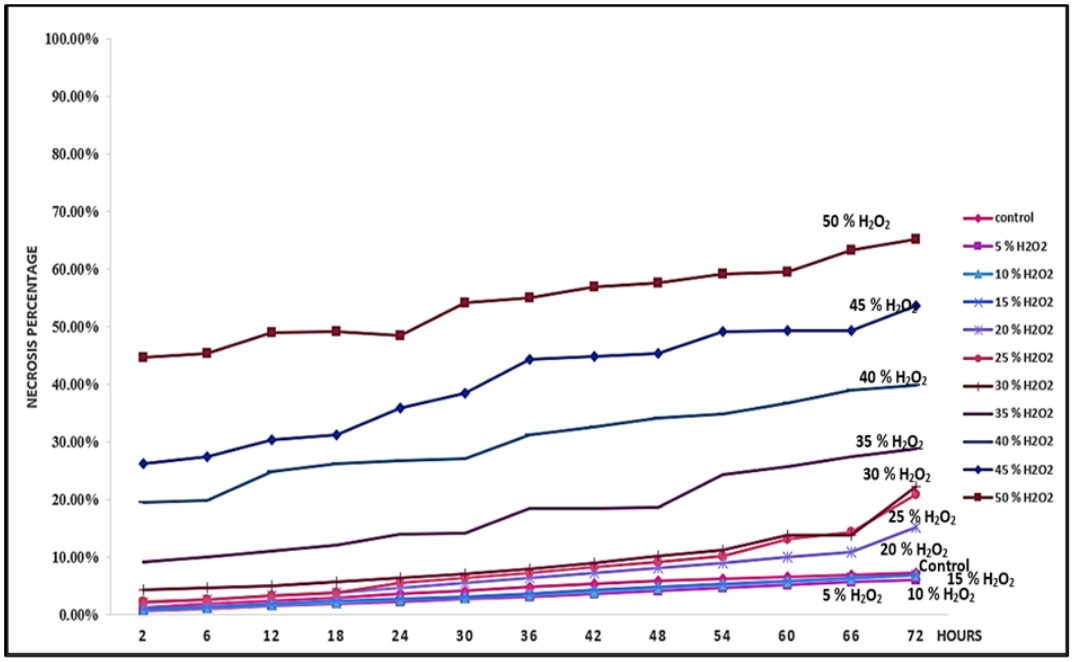

Fig. 2. Graph on the effect of different concentrations of H_2O_2 on necrosis percentage of *C. frutescens* leaf explant pre-sterilization treatment within 72 hr.

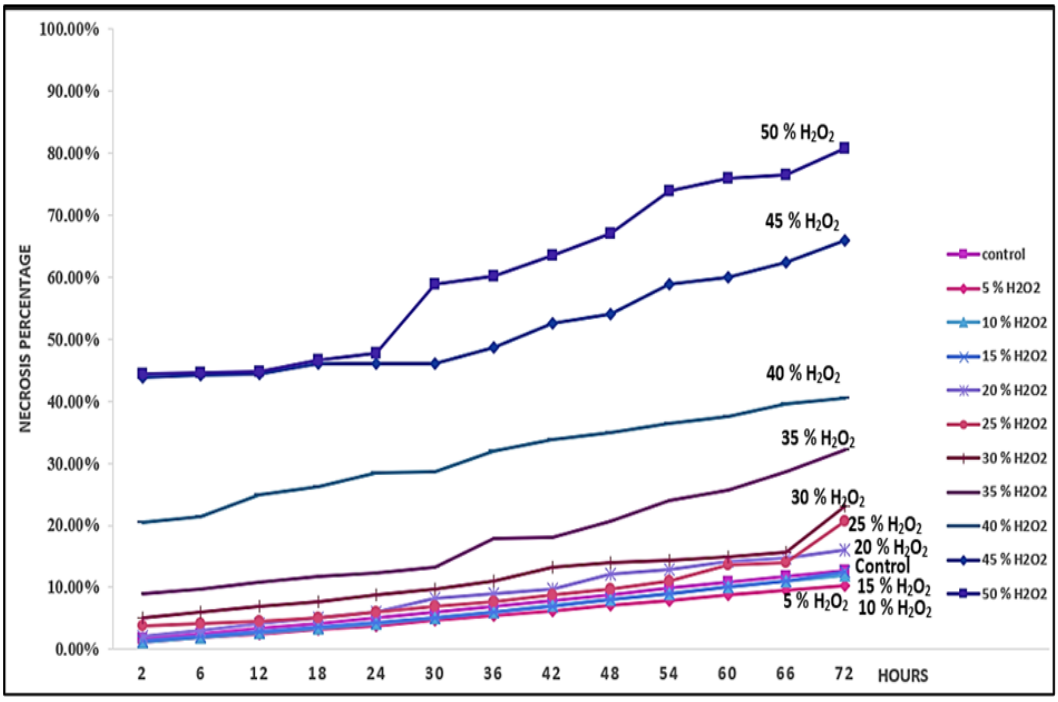

Fig. 3. Graph on the effect of different concentrations of H_2O_2 on necrosis percentage of *S. lycopersicum* leaf explant pre-sterilization treatment within 72 hr.

Furthermore, 15% H_2O_2 may have the optimal potential to increase the leaf explant survival rate for future IVC. The effectiveness of 15% H_2O_2 as pre-sterilization was shown with a clean abaxial surface of the leaf sample after optimum use of H_2O_2, which is 15% (Figure 4b & 4d) compared to previous no-application of H_2O_2 (Figure 4a & 4c). The reduction of contaminant deposition on the surface of the leaf sample was mostly caused by the abundance of free hydroxyl radicals released by high concentrations

of H_2O_2 (McDonnell, 2014; Anderson *et al*., 2024). in this case, 15% H_2O_2, which can oxidize organic matter like cellular components, cell membrane, nucleic acid, and other cell components of contaminants (Ikai *et al*., 2010; Shirato *et al*., 2012; Metwaly *et al*., 2018). The evidence of the application of high concentrations of H_2O_2 also proved to cause oxidative damage to membrane layers, proteins, enzymes, and DNA that eventually led to bacterial mortalities such as *Staphylococcus sp*., *Streptococcus sp*., *Pseudomonas sp*., and even *Bacillus* sp. endospores (Davies, 2000; Finnegan *et al*., 2010; Brudzynski *et al*., 2011).

Although early research studies had shown that the low concentrations of H_2O_2 (1%–5%) did have some degree of damage to the contaminant cell membrane, longer exposure times were required, up to 30–45 min, for H_2O_2 to be effective, yet the results showed that they were highly selective and limited (Tamarit *et al*., 1998; Cabiscol *et al*., 2000; Linley *et al*., 2012). However, in plant cells, the longer the exposure time to any liquid, disregarding its concentration, will lead to plasmolysis due to the accumulation of excess liquid. Therefore, the optimal way for pre-sterilization was to reduce the contact between the liquid (H_2O_2) and the living sample (leaf) and increase the concentrations of H_2O_2 until it's no longer causing oxidative stress with less than a 30% reduction in cell viability (necrosis) in plant cells (Luo *et al*., 2005; Anderson *et al*., 2024). Thus, 15% H_2O_2 for a min was the most optimal treatment for the pre-sterilization process for both the leaves of *C. frutescens* and *S. lycopersicum* for future IVC.

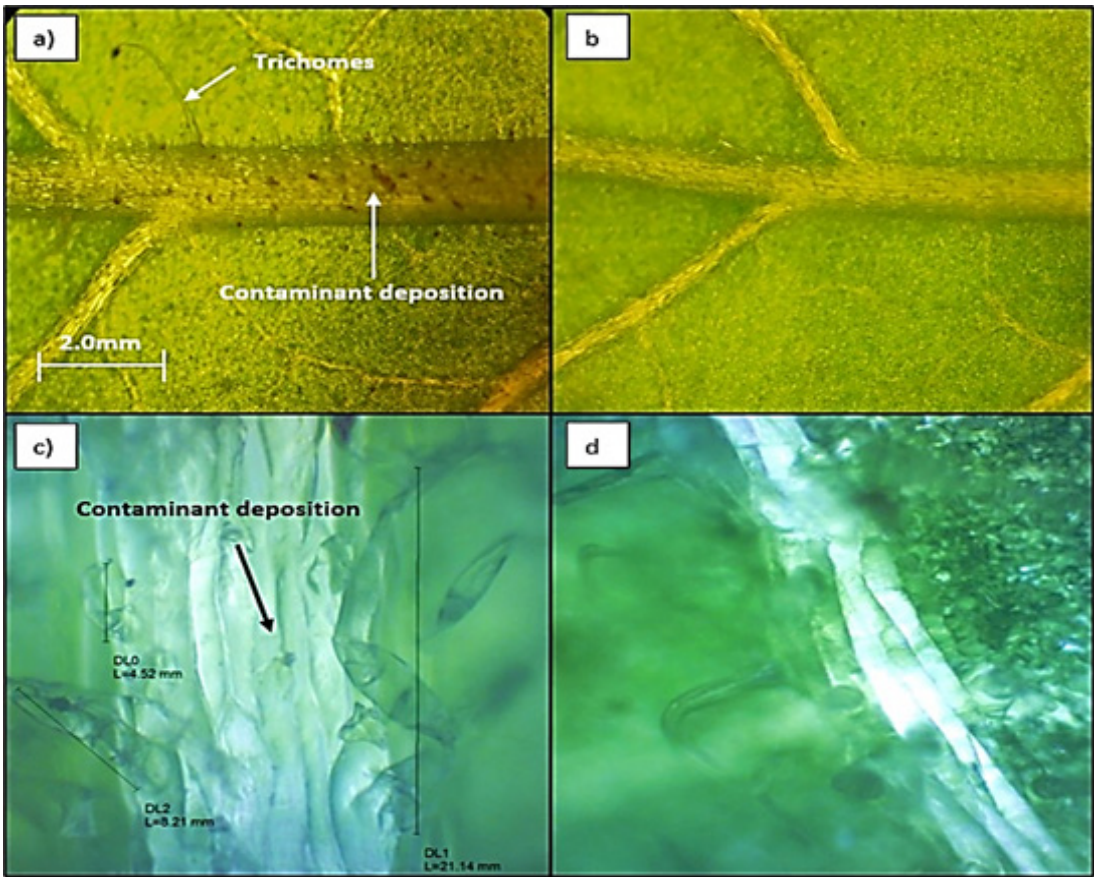

Fig. 4. Present of contaminant deposition before and after application of 15% H_2O_2 concentration as pre-sterilization on the *S. lycopersicum* abaxial leaf surface within two hr: a) 40x stereomicroscope before H_2O_2 treatment; b) 40x stereomicroscope after H_2O_2 treatment; c) 400x compound microscope before H_2O_2 treatment; d) 400x compound microscope after H_2O_2 treatment.

Analysis of sampling bag suitability for the IVC leaf sampling kit

The experiments utilized two types of vacuum-sealed bags: high-action vacuum (VSH) and low-action vacuum (VSL). The pre-sterilization step with 15% H_2O_2 from previous experiments was applied to all sampling bags as part of optimizing an IVC leaf sampling kit. The leaf samples stored at high vacuum in a VSH bag showed a wide hollow of the vacuole gap, which led to intense penetration of light from a microscopic view that provided evidence of cells in an excessive drying state for both types of leaf (Figures 5b & 5d). The cell wall and stomata were no longer visible, although the coloration of the leaf was still bright green, leading to a superficial result at the beginning of the observation for VSH

for both *C. frutescens* and *S. lycopersicum*. The VSH was able to retain the green coloration of the leaf due to a lack of oxidation in the reactive-oxidative species plant organelle inside the bag, even though there was prior application of 15% H_2O_2 (Kim & Lee, 2021). The limiting gas level is close to zero in the VSH bag, causing the cell's metabolic activity to slow down faster. On the other hand, samples in VSL received a combination treatment similar to NS and VSH, except the shortcoming of necrosis and contamination. The sample in VSL has been successfully shown to have maximum LL by retaining moisture for up to 5 days (Figures 5a & 5c).

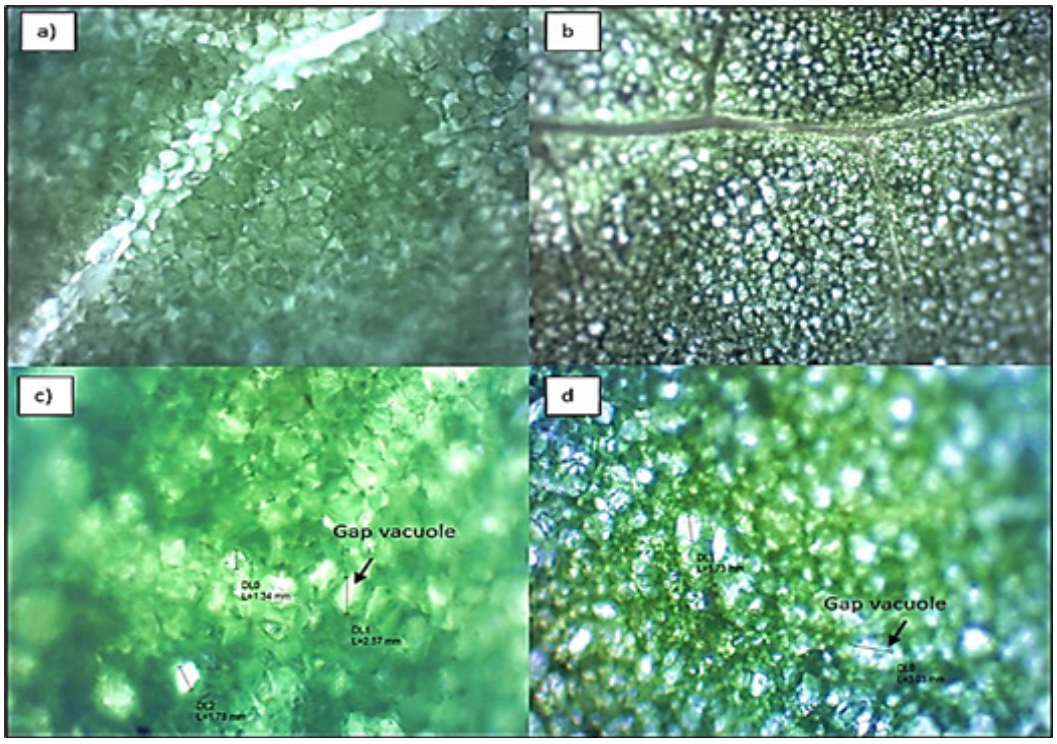

Fig. 5. Leaf cell adaxial image under 400X compound microscope after five days: a) *C. frutescens* in VSL b) *C. frutescens* in VSH c) *S. lycopersicum* in VSL d) *S. lycopersicum* in VSH

After two weeks of macroscopic observation on leaf morphology, data on various parameters were calculated and shown in Tables 1 and 2 for *C. frutescens* and *S. lycopersicum*, respectively. Results indicated that applying the optimal pre-sterilization step using 15% H_2O_2 before sealing the leaves in sampling bags significantly reduced contamination percentages in both plant species. It also extended the days before the emergence of contaminants and necrosis, resulting in

higher leaf longevity (LL) compared to samples without H_2O_2 treatment. The pre-sterilization step had a more pronounced effect on leaves in normal sealed (NS) bags than in vacuum bags (VSH or VSL), particularly for S. lycopersicum, where LL doubled from 4.78 ± 1.30 days (without H_2O_2) to 9.12 ± 2.35 days (with H_2O_2).

Controlling relative humidity (RH) was challenging due to potential gas exchange via stomata even after harvest (Gorris & Peppelenbos, 1992). High O_2 content (21% in air) within the bags could lead to oxidation by enzymes like catalase and peroxidase or bacterial aerobic activity, causing rancidity and faster decay (Maheswaran *et al*., 2021). Even though high RH promotes contamination, plants require 90–95% humidity for basic cell metabolism and 2-10% air moisture for gas exchange (Gorris & Peppelenbos, 1992; Spokowski, 2010). However, the VSH bags eliminated high O_2 content resulting in excessive drying, compromising leaf longevity despite retaining green coloration (Figure 5). In addition to the extensive contact due to high vacuum action, the VSH bag also caused permanent surface trauma on leaf surfaces, triggering more necrosis, especially in *S. lycopersicum*.

While VSL bags combine the benefits of NS and VSH bags without necrosis or contamination drawbacks, VSL samples successfully maintained moisture for up to five days, with low necrosis and reduced contamination in both species. Pre-sterilization with 15% H_2O_2 in VSL bags enhanced cell condition by at least 5% and reduced contamination to a minimum of 2%. VSL bags allowed moderate air circulation, isolating each leaf sample while permitting minimal gas exchange, similar to modified atmosphere packaging (MAP) used in the food industry (Djordjevic, 2017). This method suppressed

biological metabolism and prolonged leaf longevity by reducing oxidation speed (Maheswaran *et al*., 2021). Although specific gases were not used, minimal air was able to undergo circulation in VSL, thus extending the maximum LL compared to VSH.

Table 1. Descriptive statistic (mean ± std. dev) within two weeks of macroscopic observation on leaf morphology of *C. frutescens*

Types of sampling bag	Optimal Pre-sterilization (15%H_2O_2)	Present of contamination (%)	Days of contaminant emergence (days)	Days of necrosis emergence (days)	Maximum days of leaf longevity (days)
NS (control)	Absent	9.5% ± 2.20	3.63±0.75	4.50±1.54	4.78±1.30
	Present	4.0% ± 0.49	6.54±1.14	8.13±2.75	9.12±2.35
VSH	Absent	5.3% ± 0.47	6.19±0.90	6.93± 1.78	6.05±0.55
	Present	0.7%± 0.25	10.84±1.64	10.25±2.11	8.22±0.64
VSL	Absent	3.8% ± 0.49	7.30±1.06	7.37±0.82	8.03±0.84
	Present	0.9% ± 0.25	8.00±0.82	9.54±1.61	9.44±1.23

Notes: NS = normal sealed bag (control); VSH = Vacuum sealed bag (high vacuum action); VSL= Vacuum sealed bag (Low vacuum action)

Table 2. Descriptive statistic (mean ± std. dev) within two weeks of macroscopic observation on leaf morphology of *S.lycopersicum*

Types of sampling bag	Optimal Pre-sterilization (15%H_2O_2)	Present of contamination (%)	Days of contaminant emergence (days)	Days of necrosis emergence (days)	Maximum days of leaf longevity (days)
NS (control)	Absent	7.3% ± 0.45	3.68 ± 0.74	5.60 ± 2.50	5.67 ± 2.45
	Present	4.0% ± 0.50	5.81 ± 2.35	8.13 ± 2.75	8.50 ± 2.24
VSH	Absent	0.8% ± 0.28	6.00 ± 1.00	8.45 ± 2.63	6.08 ± 0.65
	Present	0.5% ± 0.22	11.00 ± 1.73	10.00 ± 2.24	7.45 ± 0.93
VSL	Absent	1.3% ± 0.34	8.00 ± 0.76	8.35 ± 2.44	7.87 ±1.016
	Present	0.8% ± 0.28	7.60 ± 1.14	9.93 ± 1.812	10.17 ±1.044

Notes: NS = normal sealed bag (control); VSH = Vacuum sealed bag (high vacuum action); VSL= Vacuum sealed bag (Low vacuum action)

Identification of significant differences between all the test subject variables by using one-way ANOVA during IVC treatment.

Contamination in IVC was commonly initiated by the presence of a high density of stubborn surface sterilization-resistant microorganisms with the potential for biofilm features (Umer, 2023). The use of VSH and VSL with 15% H_2O_2 had already shown an impact on reducing contamination inside the sampling bag during the collection stage. During one month on the IVC plate (Figure 5), two types of contaminants were identified for both *C. frutescence* and *S. lycopersicum,* which were bacteria and fungi. The high estimation number of a variety of contaminants present in each plate also provides a good indication of high microbial population density on the leaf surface. Another indication when the contamination emerges lags by up to seven days in either the VSH or VSL bag compared to the NS bag is that contamination is observed to emerge in less than three days.

The impact of a significant difference between the singular dependent variable (days of contamination emergence) and three independent variables was analyzed via one-way ANOVA in both *C. frutescens* (Table 3*)* and *S. lycopersicum* (Table 4). From the result, the "sample age" had significant differences in *S. lycopersicum* (*p*-value ≥ α (0.01)), but the combination of "optimal 15% H_2O_2" x "sample age" did not have significant differences at *p*-value ≥ α (0.09). Other than this, all parameters showed significant differences at *p*-values ≥ α (0.01) in both species. Therefore, the summary result showed that types of sampling bag" x "optimal 15% H_2O_2" x "sample age" had high significant differences at *p*-value ≥ α (0.01) towards days of contamination emergence in IVC in both *C. frutescens* (Table 3*)* and *S. lycopersicum* (Table 4). Since the standard practice of leaf sampling collection for IVC was lacking until now, a full “set-up” of an *in-vitro* culture (IVC) leaf sampling kit (Figure 6) by using a VSL sampling bag with pre-sterilization of optimal 15% H_2O_2 was suggested to lower the density of contamination while at the same time keeping more sustainable leaf explants while providing higher potential for successful culture in future IVC studies.

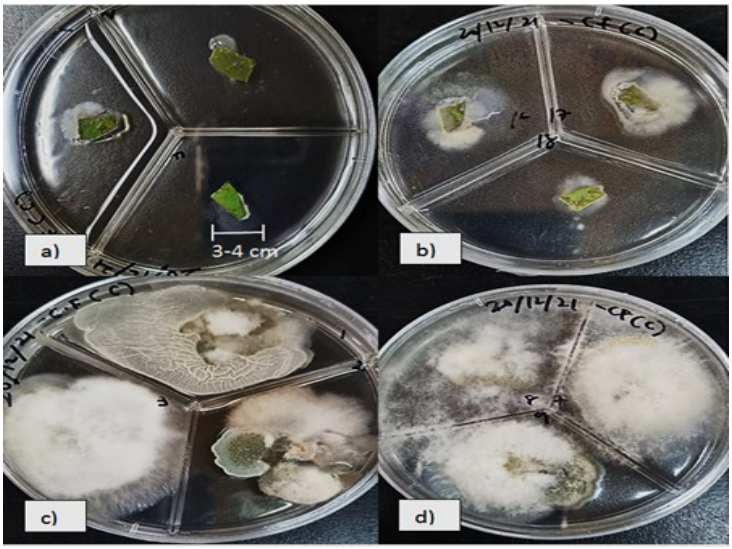

Fig. 5. Macroscopic observation on contaminant emergences in the one-month duration of IVC *C. frutescens* leaf explant; a) a week, b) 2nd week, c) 3rd week, and d) 4th week)

Table 3. Summary analysis (One-way - ANOVA) in tests of between-subjects effects of singular dependent variable (days of contamination emergence) in *C. frutescens* leaf explant towards three independent variables

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Types of sampling bag	329.136	2	164.568	356.149	<.001
Optimal 15%H_2O_2	567.113	1	567.113	1227.314	<.001
Sample Age	18.368	1	18.368	39.751	<.001
Types of sampling bag * Optimal 15%H_2O_2	193.358	2	96.679	209.228	<.001
Types of sampling bag * Sample Age	72.886	2	36.443	78.868	<.001
Optimal 15%H_2O_2 * Sample Age	7.401	1	7.401	16.018	<.001
Types of sampling bag *Optimal 15%H_2O_2* Sample Age	11.753	2	5.876	12.717	<.001

Notes: *p*-value < α (0.05) = Reject null hypothesis (H_0); *p*-value≥ α (0.05) = Accept null hypothesis (H_0).

Table 4. Summary analysis (One-way - ANOVA) in tests of between-subjects effects of singular dependent variable (days of contamination emergence) in *S. lycopersicum* leaf explant towards three independent variables

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Types of sampling bag	282.420	2	141.210	564.996	<.001
Optimal 15%H_2O_2	302.449	1	302.449	1210.130	<.001
Sample Age	3.341	1	3.341	13.369	<.001
Types of sampling bag * Optimal 15%H_2O_2	87.088	2	43.544	174.223	<.001
Types of sampling bag * Sample Age	18.547	2	9.273	37.103	<.001
Optimal 15%H_2O_2 * Sample Age	1.692	1	1.692	6.770	.009
Types of sampling bag *Optimal 15%H_2O_2* Sample Age	3.356	2	1.678	6.713	.001

Notes: *p*-value < α (0.05) = Reject null hypothesis (H_0); *p*-value≥ α (0.05) = Accept null hypothesis (H_0).

Fig. 6. Complete set–up of IVC leaf sampling kit

CONCLUSION

The study demonstrates that pre-sterilization with 15% H_2O_2 significantly reduces surface contamination and necrosis, providing an optimal balance between antimicrobial efficacy and tissue preservation. Combined with low-action vacuum-sealed (VSL) bags, this method enhances leaf longevity and minimizes contaminant emergence. Statistical analyses confirm the significant impact of sampling bag type and pre-sterilization on key parameters, addressing core challenges in plant tissue culture by minimizing microbial contamination and preserving leaf integrity. The optimized protocol significantly enhances *in-vitro* culture success rates, particularly for crops like chili and tomato, aligning with sustainability goals. Additionally, it reduces contamination rates and prolongs leaf viability, lowering costs and time for plant tissue culture, and making it more accessible for research and commercial applications. This study also opens avenues for further research on long-term impacts and optimization of storage conditions, contributing to sustainable and efficient agricultural practices.

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ETHICAL STATEMENT

Not applicable

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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