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Preserving Freshness: Alternative Collection Bags for Extended Leaf Longevity in Solanaceae Explants for In-Vitro Culture

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ABSTRACT

This study aims to determine the most effective sampling bag method to enhance sustainability by reducing contamination while preventing necrosis emergence faster during Solanaceae leaf samples (Capsicum frutescens and Solanum lycopersicum) long-distance collection for in vitro culture (IVC). By incorporating a 15% hydrogen peroxide (H_2O_2) as the initial pre-sterilization step, two types of sample bags, normal seal (NS) bags, and vacuum seal bags were evaluated based on their impact on sample viability and contamination control. The findings reveal that vacuum-sealed bags, especially those with low vacuum action (VSL), significantly extend the days of contamination and necrosis emergence during explant storage compared to NS bags and significantly prolonged the day for contamination, disregarding sample age during the IVC stage. Statistical analyses also confirm significant differences in contamination rates, necrosis, and leaf longevity between bag types and the presence of pre-sterilization methods for better outcomes. These results highlight the importance of revolutionary changes in how the IVC sample should be conducted when sampling to improve IVC success and give it better chances in future IVC and agricultural research.

Keywords: Vacuum Seal Bags; In-vitro Culture Contamination; Necrosis; Capsicum Frutescens Leaf; Solanum Lycopersicum Leaf

INTRODUCTION

Proper selection and collection methods for plant samples are crucial for achieving reliable research outcomes and establishing references for future studies. Traditional protocols for leaf plant sample collection often applied to herbarium specimens, nutritional studies, genomic research, and molecular engineering, typically require leaf samples in the dry form [1]. However, this approach contrasts with the needs of *in-vitro* culture (IVC) research, which prioritizes the maintenance of fresh leaf samples. Leaves commonly become popular due to their availability, abundance, and minimal damage to the parent plant [2]; however, they face multiple disease challenges due to environmental exposure, mainly when not cultivated indoors. Although maintaining a sterile environment [3] and regular culture monitoring are standard practices in IVC [3,4], the collection process is often overlooked. Effective protocols for sample collection and handling can prevent cross-contamination, which is an aspect that warrants more research attention. In addition, this study also seeks to identify a method that balances contamination control and sample integrity to improve the overall success rate of IVC. Two common model leaf types, the waxy adaxial leaf of *Capsicum frutescen*s and the trichome-covered adaxial leaf of *Solanum lycopersicum*, both from the Solanaceae family [5,6], are frequently used as model plants in fundamental studies due to their small size, ease of growth, small genome, and suitability for genetic transformation [7,8], while vacuum-sealed bags for collecting samples were suggested in this research.

This idea revolves around the food packaging system developed by Karl Busch in 1963. The food storage system has a primary aim to prevent oxidation by removing oxygen using high-barrier packaging materials [9-11], which helps prevent spoilage due to microorganisms [9] and consequently extends the shelf life of products [11]. Additionally, vacuum packaging in organic food studies has been proven to provide several advantages, including reduced risk of contamination [9], ease of handling [11], and inhibition of aerobic spoilage organisms [9,10]. With a few modifications, vacuum packaging may help prevent contamination and necrosis while increasing the longevity of leaf explants, ensuring they remain in excellent condition for IVC procedures, and mitigating potential explant damage. Additionally, successfully developing this sampling method for these leaf explants may provide valuable benchmarks for other IVC

leaf collection studies, especially those that are less susceptible to disease or under conservation.

The objective of this study was to determine the most effective type of sampling bag by evaluating the performance of normal seal (NS) bags versus vacuum seal bags; vacuum high-action sealed bag (VSH) and vacuum low-action sealed bag (VSL) in maintaining sample viability and minimizing contamination when combined with a pre-sterilization step using 15% hydrogen peroxide (H_2O_2). The study also aims to determine whether these pre-sterilization treatments can extend the days of contamination emergence in IVC plate in order to provide broader implications for agricultural practices, particularly in the propagation of important crop species such as *Capsicum frutescens* and *Solanum lycopersicum*. Moreover, improving sample viability and reducing contamination can lead to more efficient and cost-effective micropropagation techniques, supporting the production of high-quality plants and contributing to agricultural sustainability.

METHODOLOGY

Handling Leaf Sample Explant Source in Various Sample Bags

In this experiment, more than 25 young and healthy *Capsicum frutescens* and *Solanum lycopersicum* plants were obtained from a specific local nursery without potential diseases and were cultivated outside the laboratory. The 30 young leaf samples from both species that exhibited good condition were picked and treated with and without prior surface pre-sterilization. The pre-sterilization using 15% hydrogen peroxide (H_2O_2) was done by using foliar spray for less than a minute, then quickly rinsed with distilled water and dried adequately with tissue paper before being stored in different types of sealed bags. In normal-sealed (NS) bags, ten (10) leaves were gently placed together and immediately sealed. For the vacuum-sealed bags, the leaves were arranged without overlapping. The manual portable vacuum hand pump was used in high-action vacuum (VSH) by completely removing all air and leaving no gap. In contrast, the low-action vacuum bag (VSL) was applied carefully by leaving only small air around the leaves

but still restricting leaf movement within the bag to avoid overlapping. All bags were kept in a drying cabinet with 45% RH (relative humidity) for two weeks at 25°C.

In-Vitro Culture of Leaf Explant in Basal Media

The culture began with the preparation of Murashige and Skoog (MS) basal media by adding 4.4 g of powder to 1.0 L of sterile distilled water. After adjusting the pH to between 6.0 and 7.0, 4.0 g of agar was added to the media, which was then autoclaved at 121°C and 15 psi for 20 minutes. Leaf explants from both *C. frutescens* and *S. lycopersicum* were cut to approximately 3-4 cm before being cultured in MS media with basic sterilization procedures using ethanol and sodium hypochlorite.

RESULTS AND DISCUSSION

In obtaining plant samples at a distance, the handling of leaf samples should become a significant concern when addressing issues such as leaf longevity and contamination. Therefore, the potential use of vacuum-sealed bags (VS) compared to normal seal bags (NS) in different models of herbaceous Solanaceae leaf samples, specifically *C. frutescens* and *S. lycopersicum*, was investigated. The experiment involved two methods of using vacuum-sealed bags: high-action vacuum (VSH) and low-action vacuum (VSL). Data on leaf explant morphology, leaf longevity (LL), early contamination prevention, and necrosis emergence were analyzed after a week of observations. The results showed that normal seal (NS) bags, which had a high tendency for leaves to overlap and fold, significantly contributed to a ripple effect of contaminant infections. This caused more disturbances on leaf surfaces and led to cross-contamination when the leaves were in close contact (Figures 1 (a) and 1(b)), regardless of whether pre-sterilization with 15% H_2O_2 was applied for both species. The overlapping leaves in NS bags also caused moisture and water droplets to be trapped inside the sample bag, enhancing the route of infection [12] between leaves (Figure 2(a)). This event also led to the browning of green leaves, causing them to become rancid and start decaying, thus reducing leaf longevity or shelf life and promoting faster necrosis emergence. The high O_2 content (21% in air) in NS bags tended to oxidize by oxidizing enzymes such as catalase

and peroxidase or by further bacterial aerobic activity, contributing to this rancidification [13].

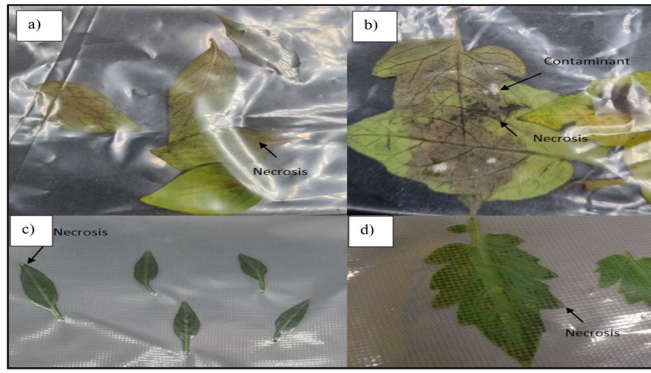

Figure 1: Comparison of leaves positioning inside two different sealed bags, normal seal bag (NS) and vacuum seal bag (VS): a) NS leaf overlapping of *C. frutescens*, b) NS leaf overlapping of *S. lycopersicum*, c) VS leaf isolation of *C. frutescens* d) VS leaf isolation of *S. lycopersicum*.

In contrast, low contamination levels were observed inside the vacuum seal bags (VSH and VSL) in both species, regardless of the presence of 15% H_2O_2. The ripple effect of contamination was avoided due to the isolation of leaf samples within the sealed bags (Figure 1(c) and 1(d)), which functioned similarly to a quarantine program during a pandemic, preventing close contact and reducing the outbreak of infections [13]. While the VSH bag may eliminate the problem of high O_2 content, which is critical to this process, the extensive contact due to the high vacuum action of the packaging material caused permanent surface trauma on leaf surfaces (Figure 2(b)). This trauma also triggered rancidity [12,14], similar to oxidation after cutting vegetables, eventually causing necrosis, especially in softer leaf tissue that lacks a waxy cuticle, like in *S. l*ycopersicum.

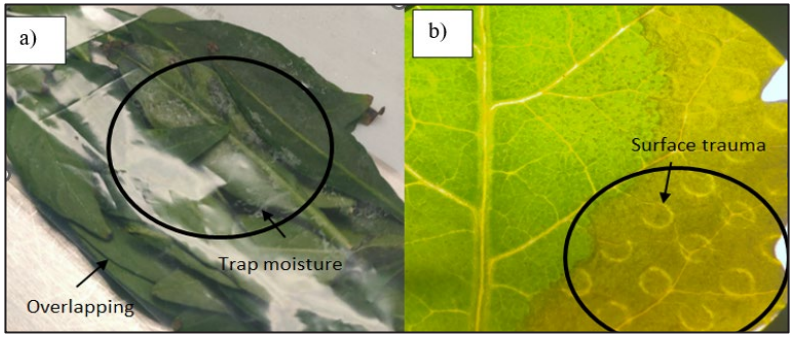

Figure 2: Two main factors led to necrosis emergence when using a) NS-overlapping and b) VSH- surface trauma.

In addition, the VSL pumped out enough moisture to isolate each leaf sample inside the bag while still allowing a moderate amount of air to circulate [10,12]. This minimal air circulation from the atmosphere allowed for more extended gas exchange [9], reducing the speed of the oxidation process. This was enough to prolong the maximum leaf longevity (LL) without injuring the leaf explant, unlike the VSH. A clear aggregation response of each parameter towards the combination of different seal bags and optimal pre-sterilization (15% H_2O_2) was graphically shown in the average response comparison (EMMEANS) in Figures 3 and 4, with a 95% confidence level of the error bar for both C. frutescens and S. lycopersicum, respectively. The graphs for both species showed almost identical values for all parameter variables except for the presence of contamination percentages (Figures 3(a) and 4(a)), where pre-sterilization widened the gap of contamination present in both VSH and VSL for *S. lycopersicum* compared to *C. frutescens.* This may be caused by the abundance of trichomes on tomato leaves compared to chili leaves, which stubbornly adhere contaminants to their surfaces [15,16], and some can only be reduced by prior extensive surface sterilization [17].

Contamination can still be present in a vacuum, as air removal may inhibit the growth of aerobic bacteria but promote the development of anaerobic [9,10] or microaerophilic-prone contaminants [10], similar to the food packaging concept. Some bacteria can survive with or without oxygen (O_2) [9]. Achieving zero or low contamination solely through vacuum action

is impossible. However, the high vacuum action (VSH) significantly reduced the O_2 content compared to the low vacuum action (VSL), which affected the days of contaminant emergence in both species (Figures 3(b) and 4(b)). The leaf samples stored at high vacuum in VSH bags showed excessive drying for both types of leaves, although the coloration remained bright green. This led to a superficial result at the beginning of the observation for VSH in both C. frutescens and S. lycopersicum. Therefore, the maximum leaf longevity of the samples in VSH was lower than in NS (with 15% H_2O_2), even with no trace of contamination and low necrosis, as shown in the EMMEANS graphs (Figures 3(d) and 4(d)).

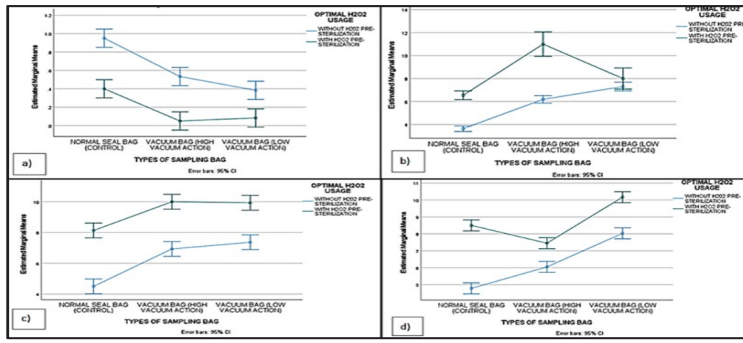

Figure 3: Estimated Marginal Means (EMMEANS) with 95% confidence level for C. frutescens a) Present of contamination; b) Days Contaminant emergence; c) Day of necrosis emergence; d) Maximum day of leaf longevity.

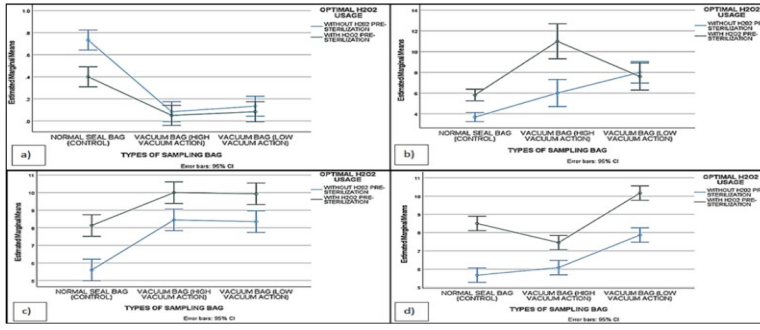

Figure 4: Estimated Marginal Means (EMMEANS) with 95% confidence level for S. lycopersicum a) Present of contamination; b) DaysContaminant emergence; c) Day of necrosis emergence; d) Maximum day of leaf longevity

The EMMEANS graph results also showed that infected leaves, even after pre-sterilization in VSL, may periodically have the same contamination emergence time frame as NS (with H_2O_2) due to the high amount of O_2 still left in the sealed bag. However, the isolation of the leaf in VSL makes it easier to discard the infected sample during IVC culture, making early days of contamination outbreaks less imperative. The longer necrosis takes to emerge, the more sustainable the leaf condition will be, thus maximizing the plant cell lifespan. The EMMEANS graphs (Figures 3 and 4) indicated that the combination of both vacuum-sealed bags (VSH and VSL) and pre-sterilization effectively extended the time taken for necrosis to emerge compared to NS. The result was also supported by the study on leafy food packaging, in which vacuum plays an important role in slowing down the browning, maintaining quality, delaying microbial growth, and extending the shelf life of fresh-cut leafy vegetables from 3 to 9 days [14].

The result from Table 1 to 4 were further analysis using MANOVA to identify significant differences between all the test subject variables. The null hypothesis (H_0), stating that traditional non-vacuum bags (NS) may be able to prevent contamination by lowering necrosis and maximizing leaf longevity, making them suitable for IVC, would be accepted at a p-value $\geq \alpha$ (0.05) or rejected at a p-value $< \alpha$ (0.05). From the result analysis in Table 1 for C. frutescens, the null hypothesis (Ho) was rejected at a p-value of 0.01 across all variables, including days of necrosis emergence, presence of contamination, and maximum days of LL, indicating a highly significant difference in using different types of sampling bags.

Table 1: Result analysis (MANOVA) in Tests of Between-Subjects Effects for *C. frutescens*

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
Types Of Sampling Bag	Days Of Necrosis Emergence	429.772	2	214.886	36.792	<.001
	Present Of Contamination	18.472	2	9.236	72.523	<.001
	Maximum Days LL	356.022	2	178.011	74.046	<.001
Optimal H_2O_2 Usage	Days Of Necrosis Emergence	321.111	1	321.111	54.979	<.001
	Present Of Contamination	1.736	1	1.736	13.632	<.001
	Maximum Days LL	422.500	1	422.500	175.745	<.001
Types Of Sampling Bag * Optimal H_2O_2 Usage	Days Of Necrosis Emergence	18.706	2	9.353	1.601	.203
	Present Of Contamination	1.706	2	.853	6.696	<.001
	Maximum Days LL	33.067	2	16.533	6.877	<.001

Notes: p-value < α (0.05) = Reject null hypothesis (H_0); p-value $\geq$ α (0.05) = Accept null hypothesis (H_0)

Table 2: Summary analysis of Multivariate Analysis of Variance (MANOVA) for *C. frutescens* in three dependent variables factors: Present of Contamination, Days of Necrosis Emergence, and maximum days of leaf longevity (LL)

Effect		Value	F	Error Df	Sig.
Types Of Sampling Bag	Pillai's Trace	.732	67.967	706.000	<.001
	Wilks' Lambda	.397	68.915[b]	704.000	<.001
	Hotelling's Trace	1.194	69.863	702.000	<.001
	Roy's Largest Root	.774	91.023[c]	353.000	<.001
Optimal H_2o_2 Usage	Pillai's Trace	.332	58.298[b]	352.000	<.001
	Wilks' Lambda	.668	58.298[b]	352.000	<.001
	Hotelling's Trace	.497	58.298[b]	352.000	<.001
	Roy's Largest Root	.497	58.298[b]	352.000	<.001
Types Of Sampling Bag * Optimal H_2O_2 Usage	Pillai's Trace	.068	4.169	706.000	<.001
	Wilks' Lambda	.932	4.176[b]	704.000	<.001
	Hotelling's Trace	.071	4.182	702.000	<.001
	Roy's Largest Root	.054	6.337[c]	353.000	<.001

Notes: p-value < α (0.05) = Reject null hypothesis (H_0); p-value $\geq$ α (0.05) = Accept null hypothesis (H_0).

Table 3: Result analysis (MANOVA) in Tests of Between-Subjects Effects for *S. lycopersicum*

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
Types Of Sampling Bag	Days Of Necrosis Emergence	404.022	2	202.011	56.250	<.001
	Present Of Contamination	13.817	2	6.908	45.456	<.001
	Maximum Days LL	463.106	2	231.553	141.534	<.001
Optimal H_2O_2 Usage	Days Of Necrosis Emergence	858.711	1	858.711	239.106	<.001
	Present Of Contamination	17.778	1	17.778	116.976	<.001
	Maximum Days LL	525.625	1	525.625	321.283	<.001
Types Of Sampling Bag * Optimal H_2O_2 Usage	Days Of Necrosis Emergence	17.089	2	8.544	2.379	.094
	Present Of Contamination	1.006	2	.503	3.308	.038
	Maximum Days LL	84.117	2	42.058	25.708	<.001

Notes: p- value < α (0.05) = Reject null hypothesis (H_0); p –value $\geq$ α (0.05) = Accept null hypothesis (H_0)

Table 4: Summary analysis of Multivariate Analysis of Variance (MANOVA) for S. lycopersicum in three dependent variables factors: Presence of Contamination, Days of Necrosis Emergence, and maximum days of leaf longevity (LL)

Effect		Value	F	Error Df	Sig.
Types Of Sampling Bag	Pillai's Trace	.767	73.156	352.000	<.001
	Wilks' Lambda	.373	74.770[b]	352.000	<.001
	Hotelling's Trace	1.306	76.390	352.000	<.001
	Roy's Largest Root	.880	103.538[c]	352.000	<.001
Optimal H_2O_2 Usage	Pillai's Trace	.561	150.060[b]	706.000	<.001
	Wilks' Lambda	.439	150.060[b]	704.000	<.001
	Hotelling's Trace	1.279	150.060[b]	702.000	<.001
	Roy's Largest Root	1.279	150.060[b]	353.000	<.001
Types Of Sampling Bag * Optimal H_2O_2 Usage	Pillai's Trace	.165	10.551	352.000	<.001
	Wilks' Lambda	.839	10.792[b]	352.000	<.001
	Hotelling's Trace	.189	11.033	352.000	<.001
	Roy's Largest Root	.165	19.467[c]	352.000	<.001

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

The combination of the application of optimal pre-sterilization (15%) H_2O_2 with the sampling bag has shown no significant difference in days of necrosis emergence (p-value ≥ α (0.203) but has shown a significant difference in the presence of contamination and prolonged leaf longevity (LL) for *C. frutescens*. Overall, the types of sampling bags and the use of H_2O_2showed a highly significant difference in all four major tests of *C. frutescens* (Table 2). Meanwhile, the result obtained for *S. lycopersicum,* with the combination of the application of optimal pre-sterilization 15% H2O2 with various kinds of sampling bags, showed no significant difference in both days of necrosis emergence (p-value ≥ α (0.094) and the presence

of contamination (p-value ≥ α (0.038), but had a significant difference in maximizing leaf longevity (Table 3). However, the final summary of the MANOVA showed that the types of sampling bags with pre-sterilization showed significant differences in all four major effects of the statistical test (Table 4).

In this section, all three types of sampling bags, NS, VSH, and VSL, were further investigated in IVC. The leaf samples were treated without pre-sterilization or with optimal pre-sterilization at 15% H_2O_2 and further aged in each type of sampling bag for ≤ 24 hours and ≤72 hours. 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 later underwent selection, where infection and necrosis were discarded before proceeding to basic sterilization. This sterilization procedure consisted of 70% ethanol and 50% sodium hypochlorite with tween 20 and distilled water rinsed before culture in MS basal media. During the duration of one month in the IVC plate (Figure 5), the days of contaminant emergences were analyzed, and the types of contaminants present were identified via macroscopic view in both *C. frutescence* and *S. lycopersicum.*

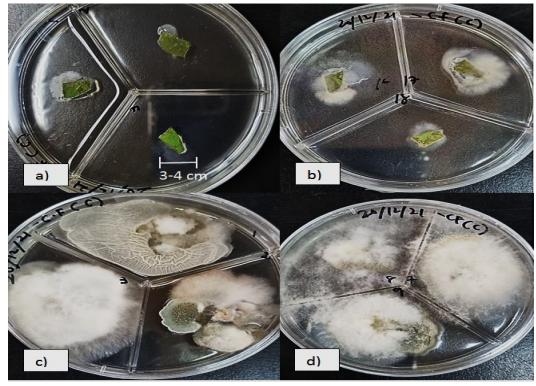

Figure 5: Macroscopic observation on contaminant emergences in the one-month duration of IVC C. frutescens leaf explant; a) a week, b) second week, c) third week, and d) fourth week)

The EMMEANS graph of *C. frutescens* (Figure 6) and *S. lycopersicum* (Figure 7) showed that the absence of optimal 15% H_2O_2 at both leaf sample ages of ≤ 24 hours and ≤72 hours caused the contaminant to emerge faster compared with the application of pre-sterilization action. In the prior experiment, 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.

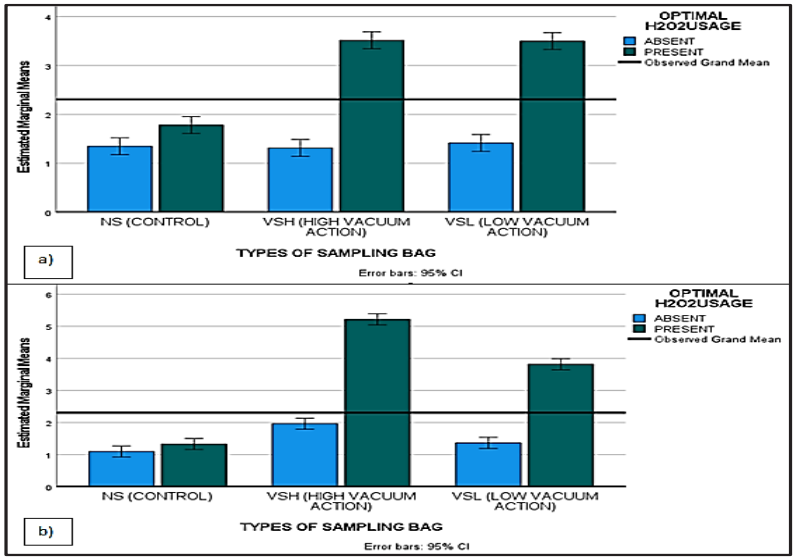

Figure 6: EMMEANS of *C. frutescens* for days of contamination emergence at leaf sample age a) ≤ 24 hours; b) ≤72 hours

Consequently, when this leaf sample underwent IVC, the contamination emerged lag by up to 4-5 days in the IVC plate, especially in *C. frutescens* explants (waxy cuticle leaf type) that had already been kept in bags for 72 hours (Figure 6(b)). Although contamination still occurs, the lag time taken for contamination to emerge may indicate a reduction in contamination density. The study on days of contamination emergence in leaf samples of *C. frutescens* and *S. lycopersicum* shows that the presence of H_2O_2 and high vacuum action (VSH) significantly delay contamination. For C. frutescens, the longest delay was observed in VSH with H_2O_2 present, reaching approximately four days at ≤24 hours and five days at ≤72 hours. Similarly, for *S. lycopersicum*, the delay in contamination was also the

longest in VSH with H_2O_2 present, reaching approximately 4.5 days at ≤24 hours and five days at ≤72 hours (Figure 7). The results of using H_2O_2 for IVC sample treatment were positively supported in previous studies that used this chemical as an antibacterial agent and were said to be more effective when combined with other antimicrobial treatments to ensure a broader spectrum of action [19-21].

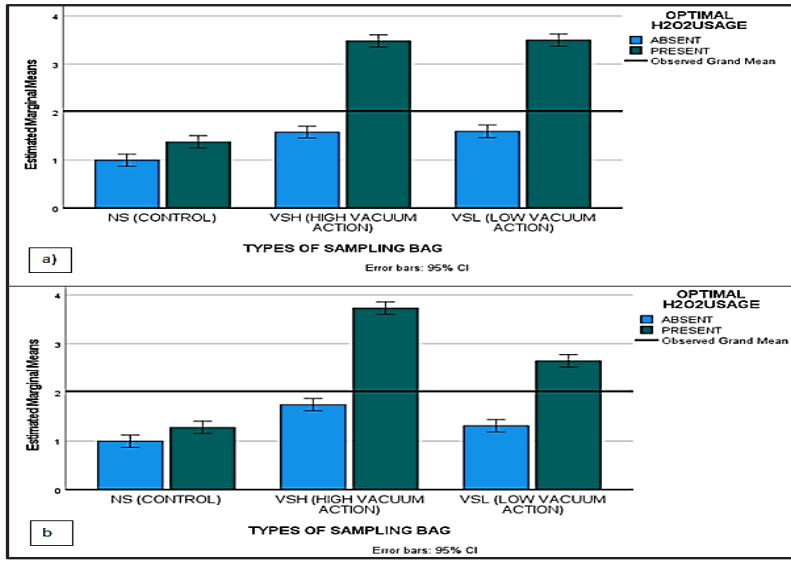

Figure 7: EMMEANS of *C. frutescens* for days of contamination emergence at leaf sample age a) ≤ 24 hours; b) ≤72 hours

CONCLUSION

This study demonstrated the significant advantages of using vacuum-sealed bags, particularly those with low vacuum action (VSL), for the collection and storage of leaf samples from *C. frutescens* and *S. lycopersicum* for in-vitro culture (IVC). The use of vacuum-sealed bags, combined with a pre-sterilization step using 15% hydrogen peroxide (H_2O_2), effectively reduced contamination and necrosis emergence compared to traditional normal seal (NS) bags and prolonged contamination during IVC. Overall, the findings suggest that implementing suitable action of using vacuum-sealed bags with appropriate pre-sterilization procedures can enhance the success rate of IVC by providing a cleaner and more controlled environment for leaf explants.

This approach not only improves the quality and longevity of leaf samples but also offers valuable insights for future agricultural and horticultural practices. These revolutionary changes in sample collection methods hold the potential to significantly improve the efficiency and outcomes of IVC and related research fields.

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