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Assessing detached onion leaves inoculated with *Alternaria porri* (Ellis) Cif. through visual and electronic means

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Abstract Spring onions are among the most valuable crops exported from Egypt. Inoculation of detached onion leaves with *Alternaria porri* (Ellis) Cif. provided an accurate and rapid method of disease severity assessment. This approach is proved superior in maintaining leaf vitality over an extended period compared to the control method. Two assessments of purple blotch disease severity were compared on detached onion leaves inoculated with *A. porri*, a quantitative assessment made via a visual assessment and a computer-based image analysis made by two raters. The image analysis provided a precise measurement of the percent lesion area of infected leaves. There was a strong positive correlation coefficient between the visual and Digital image raters (DIR) assessment (R2 =0.99). After applying the best sterilization, incubation method, detached leaf, and propagule concentration to 10^6ml *A. porri* isolates. The results showed that isolate Ay-03 had higher virulence (3.1 cm ±0.37a), (70%±12.91a), (63.5%±12.31a), (62.5%±20.81a), (100%), and (2 day±0.25a) respectively compared to the other isolates for the assessment of lesion length, disease severity (visual, rater1 and rater2), disease incidence, and incubation duration.

Keywords: Disease assessment, Detached leaf, Purple blotch, *Alternaria porri*, Spring onions

Introduction

Onion (*Allium cepa*) stands as a globally cultivated and economically significant crop, playing a pivotal role in countless culinary dishes (Cramer *et al.,* 2021). The term Allium reflects its characteristic sulfur compounds. Its taxonomical classification places in genus Allium, family Alliaceae, (Chase *et al*., 2016). Historically, onions held versatile uses, serving as medicine, herbs, spices, condiments, and even ornaments in ancient times (Li *et al*., 2010 and Hammad *et al*., 2023).

Alternaria porri is a significant fungal pathogen, poses a serious threat to onion crops, triggering purple blotch disease during winter and autumn. Study conducted in subtropical areas underscores its adverse impact on seed

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production, leading to decreased yields due to the breakage of floral stalks (Kumar, 2020). Flourishing in wet and warm climates, this pathogen significantly disrupts crucial stages such as seedling growth and flowering, causing substantial losses in both seeds and bulbs. Under favorable weather conditions, the potential loss of seed yield could reach 100% (Abo-Zaid *et al*., 2020). Symptoms of *A. porri* appear on onion leaves and flower stalks after wetting warm conditions the infected parts covered with purple colour and appeared zonation on it, characteristic of *A. porri* (Abo-Zaid, 2020).

Visual rating scales are often used to quickly assess the severity of disease in inoculated plant tissues. These scales may be percentage or numerical, with specific grades or levels. Numerical rating scales are often in 1 or 5 increments and adapted from the Keys assessment scale. Numbered grade scales, sometimes called arbitrary scales, are often employed to evaluate disease symptoms on whole plants or leaves (Couture, 1980). Accurate and reproducible disease assessment techniques are required to determine the disease response. The degree of resistance or susceptibility of onion varieties is often determined by infecting whole plants with conidia or propagules of the causal *A. porri* isolates. These inoculation trials are time-consuming and require many plants to be assessed for diseased development seven or more days after injection.

The detached leaf inoculation techniques allow testing of onion varieties for disease susceptibility without destroying whole plants, thus decreasing space needed for inoculated plants. These techniques also reduce the time between inoculation and disease assessment and may protect the environment since the pathogen is confined to the laboratory (Miller-Butler *et al*., 2013).

Software has been developed that can be used to perform very precise quantitative analysis on images of lesions on plant tissues. Although precise, the analysis software depends on a value judgment by the computer operator to determine at what level the color change on the image is considered a lesion. Digital imaging and analytical software were used to determine the percent disease and percent phytotoxicity on strawberry leaves in a miniaturized antifungal bioassay (Wang *et al*., 2008). Using Digital Imaging Techniques for assessing the severity of cucumber anthracnose disease, it caused *Colletotrichum orbiculare*. However, the duration required for capturing images of the infected tissues., utilize software to identify necrotic tissue (lesions), and determine the ratio of lesions to healthy tissue was considerable (Kwack *et al.,* 2005).

The objective was to compare computer-based image analysis of purple blotch disease on inoculated detached onion leaves with visual assessments, and to identify how well the visual assessments correlated to the computer assessment and determine if detached leaf inoculation technique could be used as primary means of estimating disease severity.

Material and methods

Growth of fungal isolates and preparation of inoculum, *A. porri* was isolated on water agar from different onion-cultivated regions in Egypt, and cultures were grown on potato dextrose agar at a temperature of 25◦C during incubation period of 7 to 14 days (Abo-Zaid *et al*., 2020).

Plant material

For this study spring onions known as Photona (*Allium cepa* L.) seeds were transplanted into pots with a mixture of soil and sand. The plants were then grown in a greenhouse until they reached the three-leaf stage. The detached leaves are sterilized using different protocols, as described below.

Sterilization methods

Freshly collected leaves 11 cm in length from the third stage leaf of plant onion and surface sterilized as follows. Freshly collected detached leaves were dipped in 70% alcohol for 1 minute: then left dry on filter paper for 5 minutes (Daud *et al*., 2012; Guo *et al*., 2016, Darojat *et al*., 2023 and Risdiyanti *et al*., 2023). The plant materials were surface sterilized using a locally available bleach solution 10%Clorox® (5.25% sodium hypochlorite) for 1 minute; then left dry on filter paper for 5 minutes (Daud *et al*., 2012; Dumin *et al*., 2021 and Darojat *et al*., 2023). The detached leaves were surface treated in 30%H_2O_2 for 1 minute; then left dry on filter paper for 5 minutes (Hong *et al*., 2013 and Ahmadpoor *et al*., 2022).

Surface sterilization using a combination of ethanol 70%, 10%Clorox® (5.25% sodium hypochlorite), then 30%H_2O_2 for 20 seconds/ each respectively; these were left dry on filter paper for 5 minutes (Ahmadpoor *et al*., 2022 and Darojat *et al*., 2023). Control was treated with water sterilized for 1 minute, then left dry on filter paper for 5 minutes.

Media

Agar media method: Onion-detached third mature leaf surface sterilization with 6 detached leaf replicates and put every two detached leaves on water agar in a petri dish (12cm) were done (Imathiu *et al.,* 2009; Guo *et al*., 2016 and Bhattarai *et al*., 2020).

Filter paper method: Onion-detached third mature leaf surface sterilization with 6 detached leaf replicates and put every two detached leaves on

filter paper in a petri dish (12cm) were done (Jacobs *et al.* ,2008; Bhattarai *et al*., 2020 and Darojat *et al*., 2023).

Chlorosis assessment

The collected leaves of onion with different ages (first, second, third mature leaf) and 11 cm in length (6 replicates for every stage) were treated with 10% Clorox put on agar media to measure their effect on leaf chlorosis.

Pathogenicity and disease assessment on detached onion leaves

Conidial suspensions used for inoculations were prepared from 7- to 14-day-old cultures. Inoculum was prepared by flooding each culture plate with sterile deionized water containing 0.5 ml Tween® 20 per liter as a surfactant and gently scraping the agar surface with a glass rod to remove conidia. The resulting conidial suspension was filtered through cheesecloth and adjusted to a concentration of 1×10^6 propagule /ml by diluting with sterile distilled water containing Tween® 20. The collected leaves of the onion in different ages (first, second, third mature leaf) 11 cm in length (6 replicates for every stage), sterilization and with 10% Clorox put on water agar media inoculated with 10µl suspension from *A. porri* isolate different concentration propagule (1×10^4, 1×10^5 and 1×10^6) to measure the disease severity on detached leaves of different stages using illustration Keys assessment (Numbered grade scales, Table 1) (Chaerani *et al*., 2007 ; Imathiu *et al.,* 2009 and Abo-Zaid *et al*., 2020) and Digital ImageJ 1.54 d. Program (Rater1: IsoData, Red, HSB)and (Rater2: IsoData, B&W, HSB) (Miller-Butler *et al*., 2013).

Table1. Disease rating scale used for disease severity of *A. porri* on onion leaves

Scale rating	Disease severity (percentage)
0	No disease symptom
1	Spot toward tip covering from 1-15 percent of leaf area.
2	Brown patches and discolor cover 16-25 percent of the leaf area.
3	Patch with paler outer zone increase covering from 26- 35 percent of leaf area.
4	patch with a paler outer zone covering 36-45 percent of the leaf area
5	Leaf covering up to 50 percent of leaf area and converting yellowish leaf

Scale of disease severity (Pandey *et al*., 2003 and Abo-Zaid, 2020) used for calculated in formula Percentage of DS:

$$\text{Percent of D.S.} = \frac{\sum (\text{class rating} \times \text{class frequency})}{\text{Total number of samples} \times \text{highest rating}}$$

Statistical analysis

The statistical software SAS 2006 was used to compute the data and present the results as means and standard errors. The Multiple Range Test of Duncan (Duncan, 1955) was used to make comparisons.

Results

Sterilization methods and incubation media

Onion-detached leaves were used in different sterilization experiments. Continuous monitoring was carried out for six days while detached leaves were in agar media or filter paper. The best sterilization of separated onion leaves was 10% Clorox, whether on agar media or a filter paper method, as the rate of yellowing of the leaves (0%) was less than experiment 3 H_2O_2 (6.67%), experiment 1Ethanol 70% (33.33%) and experiment 4 Mix (43.33%) on agar media method, respectively (Figure 1). As for the filter paper method, there was a percentage of yellowing in the leaves from lowest impact to highest was Clorox (26.67%), H_2O_2 (26.67%), Ethanol 70% (33.33%) and Mix (46.67%) (Figure 2).

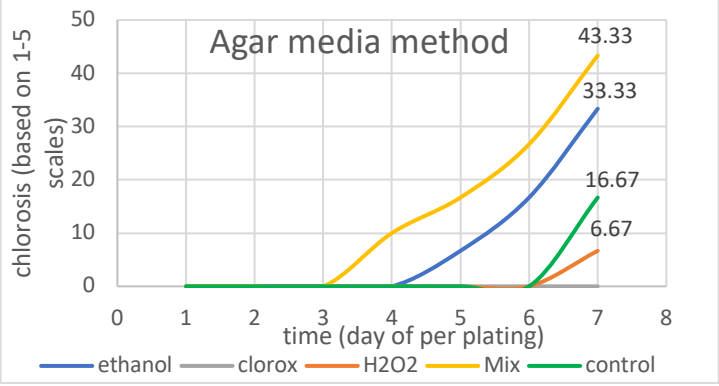

Figure 1. Effect of agar media method and type of sterilization on maintaining green color in detached leaves of spring onion. Leaves were rated for percentage of green color using a1 to 5 chlorosis scales in which 1= 0:5%chlorosis, 2= 5:20% chlorosis, 3= 20:50% chlorosis, 4= 50:80% chlorosis and 5= 80:100% chlorosis.

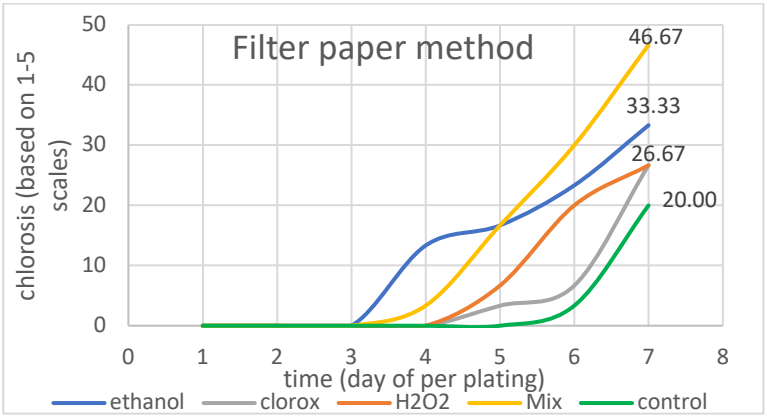

Figure 2. Effect of filter paper method and type of sterilization on maintaining green color in detached spring onion leaves. Leaves were rated for percentage of green color using a1 to 5 chlorosis scales in which 1= 0:5%chlorosis, 2= 5:20% chlorosis, 3= 20:50% chlorosis, 4= 50:80% chlorosis and 5= 80:100% chlorosis.

Chlorosis assessment

Clorox and agar media method were chosen as the best sterilization and incubation methods to determine their impact on different ages of detached leaves (leaf1, leaf2, and leaf3) to ensure that showed no chlorosis occurring on onion-detached leaves. It was determined the optimal approach for inducing disease severity on detached leaves by assessing the efficacy of sterilization, preservation methods, and their compatibility with leaf age.

Pathogenicity and disease assessment on detached onion leaf

Pathogenicity test on wounded leaves was used a composite inoculum of *A. porri* isolate different in concentration propagule ($1\times10^{\wedge4}$, $1\times10^{\wedge5}$ and $1\times10^{\wedge6}$) to measure disease severity on detached leaves of different ages (leaf1, leaf2 and leaf3). It involved in creating wounds on detached leaves. This facilitated the entry of fungi that might not penetrated to the cuticle during a natural infection, allowing them to surpass this barrier. The successful infection and disease progression are caused by pathogenic fungi, whether with or without the wounds, became evident through symptoms such as the appearance of discolored, deformed, necrotic, or chlorotic areas on detached leaves.

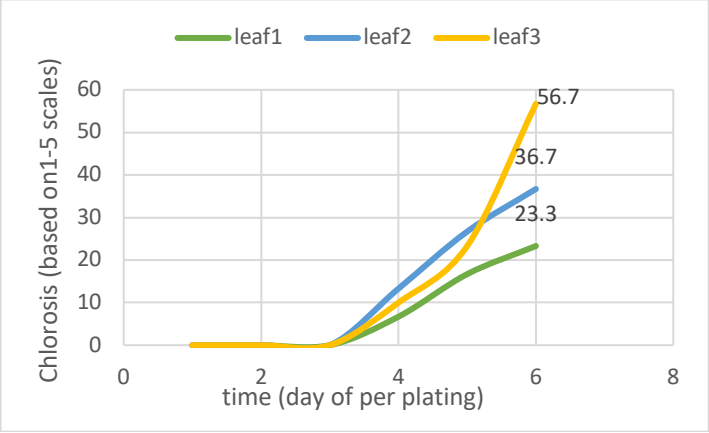

Figure 3. Effect of leaf age on marinating green color in detached leaves of spring onion on agar media method: Leaves were rated for percentage of green color using a1 to 5 chlorosis scales in which 1= 0:5%chlorosis, 2= 5:20% chlorosis, 3= 20:50% chlorosis, 4= 50:80% chlorosis and 5= 80:100% chlorosis

The tested fungi caused blotches on detached wounded leaves of onion by the sixth day post inoculation at 25°C. No lesions were observed on control leaves. blotch, almost shape oval, appeared as water-soaked lesion characterized by necrosis and chlorosis on onion leaves at 25°C. The result was found to be significant in leaf 3 at concentration propagule 1×10^6 (3.83 cm) and (64.85%) compared with other results shown in Figure 4.

Purple blotch symptoms on onion detached leaves were observed after inoculation with *A. porri*. The original photograph of *A*. *porri*-inoculated leaf (visual) and the digitally enhanced versions by two raters (rater1 and rater2) were examined. Photographs of the inoculated detached leaves clearly displayed lesions (Figure 5), and even without digital analysis, these images were suitable for visual scoring at a convenient time for each rater. Computer analysis of the photographs successfully identified the blotch, providing consistency among observers who assessed the leaves in the laboratory. The rater1 and rater2 illustrated the difference between an original leaf photograph and the same photograph after computer enhancement. Backlighting the leaflets in the photograph enhanced the blotch areas by intensifying the contrast between healthy and diseased tissue. It improved the raters' ability to observe the disease incidence more clearly compared to viewing the leaves under laboratory lights.

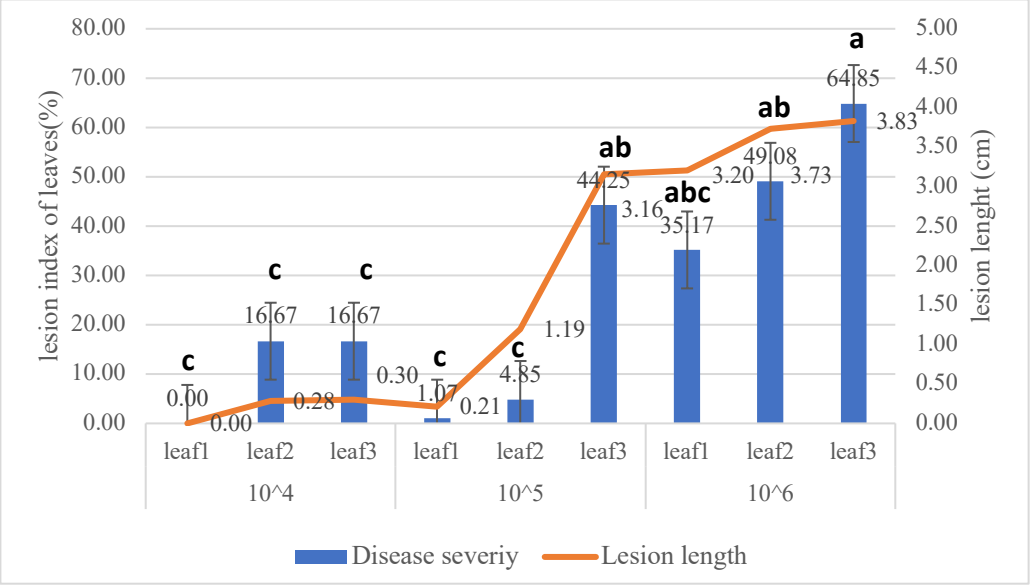

Figure 4. Effect of leaf age and different concentration of *A. porri* inoculum (propagule/milliliter) on lesion measuring (cm) and disease severity (%) on agar media

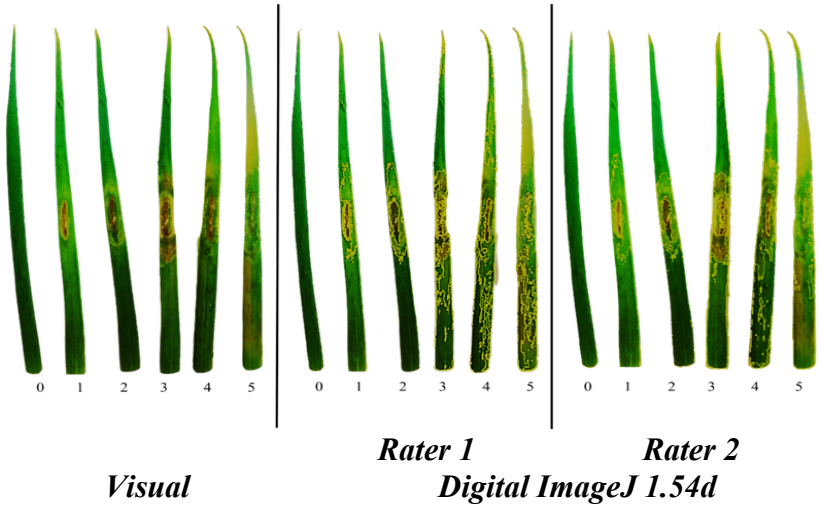

Figure 5. Disease severity assessment on detached Leaf area (0-5) scales visual and Two Raters used Digital ImageJ 1.54d program. used a scale of 0–5 (0=no disease and 5=most severe disease) to assess the percent diseased leaf area Numbered grade scales, sometimes called arbitrary scales, are often employed to evaluate disease symptoms on whole plants or leaves and are adequate wounded detached leaves 6 days post-inoculation at 25°C.

The results of the three methods are employed to assess disease severity indicated in Figure 6 which illustrated a correlation coefficient between disease severity and various disease assessment methods (R^2=0.9919).

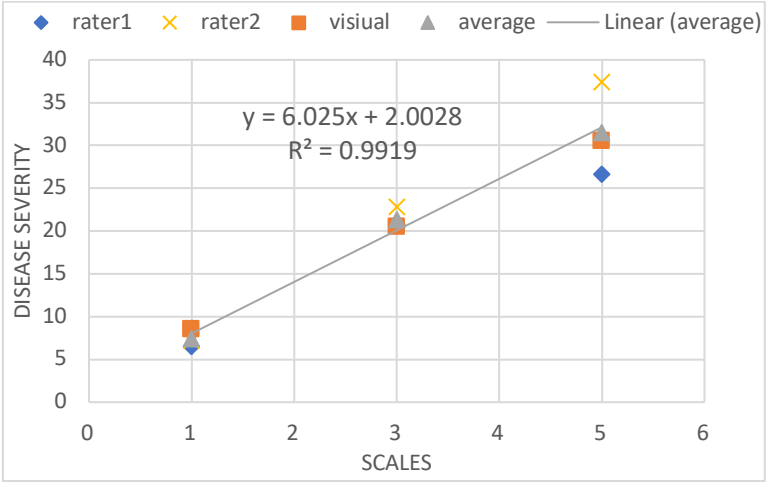

Figure 6. Correlation coefficient between disease severity and different disease severity assessment methods

The onion-detached leaves sterilized with NaOCl on water ager media and infection with 10 isolates from different isolation areas in Egypt was shown in Table 2, it referred to the incubation period between isolates being non-significant; the lesion length of Ay-03 (3.1 cm), the isolate was significant with other isolates, except Ay-04 (2.4 cm), Ay-05 (2.5 cm), and Ay-07 (2.4 cm). The disease incidence of isolates referred to Ay-01, Ay-02, Ay-03, Ay-04, Ay-05, Ay-06, and Ay-07 which caused the disease incidence at 100% in all replicates; Ay-08 andAy-10 caused disease incidence at 75% in all replicates and Ay-09 caused disease incidence at 50% in all replicates. The observed disease severity percentages (visual) were Ay-03 (70%), Ay-05 (65%), Ay-06 (65%), Ay-07 (60%), Ay-04 (55%) Ay-01 (45%), Ay-02 (40%), Ay-08 (40%), Ay-10 (30%) and Ay-09 (15%) were significantly differed. The observed disease severity percentages (Rater1) were Ay-06 (67.6%), Ay-03 (63.5%), Ay-05 (61.4%), Ay-04 (58.7%), Ay-07 (51.1%) Ay-01 (50.6%), Ay-02 (33.5%), Ay-10 (33.3%), Ay-8 (24.7%) and Ay-09 (1.0%) were significantly differed, and percentages of disease severity with Rater2 had no significant difference.

Table 2. Disease reaction following inoculation with different isolates of *A. porri* in detached leaf assay of spring onion

Isolates	Detached leaf assay (Agar media method)					
	Incubation (day)*	Lesion length (cm)	Incidence (%)	Severity (%)		
				Visual [1]	Rate1[2]	Rate2[2]
Ay-01	3±0.48▪[a]	1.6± 0.38[bcd]	100	45±15.0[ab]	50.6±18.07[a]	29.1±22.49[a]
Ay-02	3±0.50[a]	1.4±0.25[bcd]	100	40± 20.0[ab]	33.5±16.18[ab]	25.4±18.02[a]
Ay-03	2 ±0.25[a]	3.1±0.37[a]	100	70±12.91[a]	63.5±12.31[a]	62.5±20.81[a]
Ay-04	2±0.25[a]	2.4±0.47[ab]	100	55±22.17[ab]	58.7±20.44[a]	51.0±18.05[a]
Ay-05	2±0.29[a]	2.5±0.30[ab]	100	65±9.57[ab]	61.4±6.97[a]	44.6±14.23[a]
Ay-06	3±0.48[a]	2.0± 0.39[bc]	100	65± 20.62[ab]	67.6±22.87[a]	54.8±13.80[a]
Ay-07	2±0.25[a]	2.4±0.11[ab]	100	60±8.16[ab]	51.1±11.23[a]	58.6± 16.0[a]
Ay-08	3±0.82[a]	0.8±0.32[d]	75	40±18.26[ab]	24.7±18.96[ab]	20.3±16.36[a]
Ay-09	3± 0.87[a]	0.6±0.32[d]	50	15±9.57[b]	1.0±0.60[b]	2.0±1.48[a]
Ay-10	3±0.82[a]	1.0±0.35[cd]	75	30± 17.32[ab]	33.3±19.57[ab]	27.9±23.15[a]
control	0.0[e]	0.0[e]	0.0	0.0[e]	0.0[e]	0.0[e]

***** Incubation period refers to the time from the initial injection of *A. porri* until the appearance of symptoms, ▪ standard error, [1] Disease assessment keys method, [2] Disease assessment with used Digital ImageJ 1.54d.

Discussion

The study identified the best sterilization methods for detached onion leaves and the most effective incubation methods for preserving them for an extended duration. Sterilization with Clorox was the best to separate leaves compared to other treatments, A widely used sterilizing agent for disinfecting various plant types was bleach, and it observed that a 1.4% NaOCl solution for 1 minute was efficiently found in sterilized leaves, as demonstrated with *A. crasna* and *A. sinensis* sourced from greenhouses (Okudera and Ito, 2009). Hassan *et al.* (2011) who achieved the effective sterilization by using 50% bleach for 20 minutes on shoot tips and nodal explants obtained from young seedlings of *A. hirta*, and also sourced from a greenhouse. However, the study did not provide the percentage of 'clean and alive' explants. Three different methods of surface sterilization (Ethanol, Sodium hypochlorite, and combination) were assessed using seeds and excised embryos of cowpea, rice, and sorghum as explants. Our findings revealed that using the water agar incubation method and Sodium hypochlorite sterilization protocol yielded the highest reduction in bacterial and fungal contamination (0%) within time intervals ranging from 20 to 45 minutes. These experiments were found to be a simple, rapid, and cost-effective method for sterilizing explants in tissue culture, as an alternative to the conventional two-

step, and two-reagent technique. Consequently, it is recommended that this technique is shown to be simplicity and cost-effective (Oyebanji *et al*., 2009).

Various sterilization agents were assessed for disinfecting chinaberry leaf explants. The results revealed that the most favorable outcomes, including the lowest percentage of explant contamination and browning along with the highest percentage of callus induction and callus growth, were achieved when explants were pretreated with benomyl (2 g/L) for 2 hours and then sterilized with 7% H_2O_2 for 10 minutes and NaOCl 2% (without pH adjustment) for 12 minutes. While adjusting the pH of NaOCl to pH=7 and 10 significantly reduced microbial contamination and increased the percentage of contamination-free cultures for *M. azedarach* L., it had an adverse impact on explant viability as well as callus induction and growth (Ahmadpoor *et al*., 2022). It is clearly seen that chlorine does not have a harmless effect when used to sterilize plant parts.

Comparisons were made among sodium hypochlorite (NaOCl) and mercuric chloride (HgCl2) surface sterilization, methyl bromide and propylene oxide fumigation, and gamma irradiation treatments to assess their effectiveness in eliminating microorganisms on or within barley seeds. Surface sterilization using 12.5%, 25%, or 50% (v/v) NaOCl for 5, 15, or 30 minutes resulted in a reduction of *Fusarium* spp., *Epicoccum purpurascens*, and *Bacillus* spp., but was ineffective in eliminating *A. alternata* (Ramakrishna *et al.,*1991). This previous study indicated that NaOCl does not affect the *Alternaria* leading to no effect to propagule when pathogenicity on onion detached leaves.

In our study, the best propagule concentration of 10^6 was employed to initiate disease severity on detached leaves. Isolates A1-03-04 and B-62-04 of *A. porri* , capable of conidia production were inoculated using a 20 µL aqueous spore suspension at a concentration of 10^6 conidia mL^{-1}. It used to report that the inoculated fruits were placed in a moist chamber at 25ºC under a 12-hour photoperiod. After 12 days, symptoms of Alternaria Brown Spot (ABS) were observed, and the pathogen was re-isolated. The frequency of plant pathogenic fungi was quantified as the percentage of fruits where the fungus was isolated and pathogenicity was confirmed (Carvalho *et al*., 2008). Elevating the concentration of *Alternaria* Leaf Blight inoculum from 10^2 conidia to 10^6 conidia/ml resulted in a significant increase in infection. The disease intensity was minimal at 5×10^2 conidia/ml. For successful infection, an inoculum of 1 x 10^6 spores/ml and 25-30-day-old plants proved to be ideal. The greenhouse assay provided more reliable and consistent measurements of resistance to *A. helianthi* in sunflower compared to the detached leaf assay (Prasad *et al*., 2008).

In another study, Rye grains provided optimal mycelial growth for *A. porri*, while good mycelial growth was observed on onion leaves, onion seed stalks, onion bulbs, and garlic leaves. Poor mycelial growth was noted on onion seeds and maize grains. Among the eight natural substrates tested, only onion

leaves and onion seed stalks were found to support the sporulation of *A. porri*. None of the other natural substrates were conducive to the sporulation of the fungus. Notably, onion seed stalks supported significantly higher sporulation (8.26×10^5 conidia/ml) as compared to onion leaves (2.80×10^5 conidia/ml) based on Tukey's HSD test ($P \leq 0.05$) (Yadav *et al*., 1970).

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