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GENOTYPE-DEPENDENT RESPONSES TO STERILIZATION AND RESTERILIZATION IN *IN VITRO* CULTURES OF *HYDRANGEA MACROPHYLLA*

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ABSTRACT

Establishing axenic cultures of *Hydrangea macrophylla* remains a significant challenge due to persistent endogenous contamination. This study evaluated complex disinfection and resterilization protocols for three cultivars:"Love", "Tivoli®", and "Masja". Explants (apical/lateral buds) were subjected to multi-step sterilization involving ethanol, sodium dichloroisocyanurate (NaDCC, 0.2 - 2%), permanganate, and fungicidal (Dithane®) or bleach-based disinfectant (Domestos®) - commercial bleach. Inoculations were done on MS medium with BAP or BAP + kinetin, and cultures were maintained at 23 - 25°C, 2000 - 2500 lux, 16 h photoperiod. Initial contamination exceeded 80% for all genotypes. Weekly resterilizations (up to three cycles) using NaDCC (2%) + 3 drops Domestos reduced bacterial and fungal contamination by ~45% in 'Masja' and ~35% in "Love", but only marginally (~10%) in "Tivoli®". Fungal suppression was more effective when Dithane® (0.2%) was included for 7 minutes prior to NaDCC treatment. "Tivoli®" explants exhibited persistent internal bacterial symptoms despite surface sterilization, confirming its status as the most recalcitrant genotype. Survival and meristem establishment rates were highest in "Masja" (34%) and lowest in "Tivoli®" (8%). Callus induction occurred in up to 40% of clean explants. Multiple rinses (3×2 min in sterile water) and intermediate permanganate exposure (max. 15 minutes) proved critical in improving aseptic conditions.

The study demonstrates that decontamination efficiency is highly genotype-dependent and supports a tailored, iterative approach to resterilization. Results provide practical guidance for establishing stable *in vitro* cultures of *Hydrangea macrophylla* cultivars for conservation and micropropagation.

Keywords*: Contamination, Meristem culture, Disinfection protocol.*

INTRODUCTION

Hydrangea macrophylla (Thunb.) Ser., commonly known as bigleaf hydrangea, is a highly valued horticultural species cultivated globally for its large, ornamental inflorescences and striking morphological variability. It belongs to a genetically diverse lineage comprising over 200 species within the genus Hydrangea sensu lato (Hydrangeaceae), which is widely distributed across Asia and the Americas (*Ishiguro et al., 2025).* Recent studies have also highlighted the species' potential invasiveness in certain regions. For instance, Hydrangea macrophylla is classified as an alien invasive species in the Azores and Madeira archipelagos, where it has been associated with adverse impacts on native habitats and local biodiversity, as has been the case with other introduced ornamental species in other countries. (Raicu et al., 2024; Life Beetles Azores, 2022). This lineage encompasses not only cultivated forms but also wild relatives and interspecific hybrids, all contributing to the extensive phenotypic plasticity observed across the group (Sevilleno et al., 2025). The remarkable variation in floral architecture, pigmentation patterns, leaf morphology, and growth habit is mirrored by substantial genomic diversity, including differences in genome size, base chromosome number, and ploidy level among taxa and cultivars. Its propagation through conventional means (e.g., stem cuttings or seed) is often constrained by genotype-specific rooting responses, genetic variation in progeny, and pathogen carriage, limiting clonal fidelity and scalability (Nagashima et al., 2021; Anikina et al., 2025).

In vitro micropropagation is thus regarded as a crucial biotechnological tool for *H. macrophylla*, enabling rapid, large-scale, and true-to-type multiplication under controlled and ostensibly sterile conditions (Kitamura et al., 2008; Abdalla et al., 2022). However, initiation of *Hydrangea* cultures frequently fails due to persistent endogenous microbial contamination**,** especially by internal bacteria and fungi that survive surface sterilization and proliferate during culture establishment. (Cassells, 1997)

Effective surface sterilization is a critical step for ensuring successful in vitro establishment of plant tissue culture. Classical sterilants such as mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) are widely used due to their broad-spectrum efficacy, but their impact varies depending on species, explant type, and concentration (e.g., 0.15% $HgCl_2$ for 8 min yielded 100% survival in Gloriosa superba). A comparative study on water lily found that $HgCl_2$ achieved higher decontamination rates and less explant browning compared to NaOCl, highlighting the balance required between sterilization power and tissue viability (Lin et al., 2025). Recently, sodium dichloroisocyanurate (NaDCC) has emerged as a non-toxic, stable, chlorine-releasing agent, effective for explant and medium sterilization in multiple ornamental species at concentrations between 0.05 - 1 g L^{-1} (Simran & Narasimhan, 2021); Kabadayi et al., 2024). Its use provides sterilization comparable or superior to NaOCl, with fewer phytotoxic effects and environmental risks. Genotype-specific responses to sterilants have been documented: certain Hydrangea cultivars may require $HgCl_2$ for effective decontamination, while others tolerate milder chlorine-based treatments, consistent with broader findings that species and

genotypic traits influence explant sensitivity and microbial load (Khaing et al., 2018; Doil et al., 2008; Anđelić et al, 2024).

Despite the widespread relevance of these sterilants in ornamental micropropagation, there is a notable research gap regarding genotype-level comparisons in Hydrangea macrophylla (Doil et al., 2008, Šiško M., 2016). Many protocols focus on shoot proliferation or rooting (e.g., MS medium with BA or IBA), but seldom correlate sterilant combinations with contamination outcomes among different cultivars. This study aims to fill that gap by systematically evaluating sterilization profiles - combinations of fungicide, permanganate, $HgCl_2$, NaDCC, and bleach - across three cultivars ("Tivoli", "Masja", and "Love"), with statistical analysis of disinfection efficiency and genotype variability.

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MATERIALS AND METHODS

Three *Hydrangea macrophylla* cultivars ("Tivoli", "Masja", "Love") were included in this study, originating from the "Alexandru Ciubotaru" National Botanical Garden in Chișinău. Nodal buds, shoot tips (meristems), and already cultivated *in vitro* shoots were surface disinfected using various combinations of five agents: a commercial fungicide (Dianthe®) (F), potassium permanganate (P), mercuric chloride ($HgCl_2$) (H), sodium dichloroisocyanurate (NaDCC), and Domestos® bleach based on NaOCl (D). Treatments involved 1 to 5 agents, applied sequentially, with exposure times between 2 and 10 minutes. Concentrations and protocols were adjusted based on preliminary tolerance and re-sterilization needs.

All explants were cultured on Murashige and Skoog (MS) medium with 3% sucrose and 0.7% agar. Contamination was visually evaluated at 5 days after inoculation and usually at 2 - 3 weeks, and explants were classified as clean or contaminated. Contaminated explants were re-sterilized and reassessed.

Disinfection treatments were coded as binary profiles (presence/absence of F, P, H, N, D), allowing systematic comparison between genotypes. For each profile, contamination-free percentages were calculated and analyzed. Differences between cultivars were tested statistically using Pearson's Chi-square test. Highly significant variation (e.g., $p < 0.0001$) showed that genotype-specific responses were relevant. Data processing and plotting used Python libraries. A co-occurrence matrix was generated from pairwise and triplet frequencies across treatments. Frequency tables and heatmaps were used to identify recurring combinations and assess potential procedural standardization or redundancy.

RESULTS AND DISCUSSION

Analysis of disinfection protocols by explant type revealed significant variation in treatment complexity and agent usage. On average, meristem cultures received the highest number of disinfection agents per treatment (3.14), followed by buds (2.67),

and *in vitro* shoots (1.71). Meristem cultures consistently included potassium permanganate (100%), mercuric chloride (86%), and Domestos® (100%), with fungicide applied in 29% of cases, but never NaDCC. In contrast, *in vitro* shoots were treated exclusively with NaDCC (100%) and Domestos® (71%), without any use of $HgCl_2$, permanganate, or fungicide. Buds received a more balanced profile, with moderate frequencies for each agent: permanganate and $HgCl_2$ (67% each), Domestos® (61%), NaDCC (44%), and fungicide (28%). The results show that disinfection procedures were adjusted according to explant type, with stronger chemical treatments consistently applied to meristematic tissues, which have a higher risk of internal contamination.

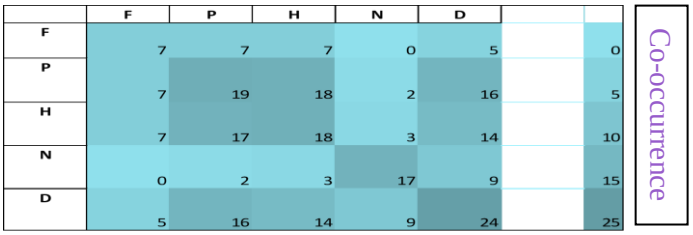

Figure 1. Heatmap of disinfectant co-application frequencies in *Hydrangea macrophylla*.

A co-occurrence matrix (Fig. 1) was constructed to quantify the frequency of joint application of five disinfectant agents: fungicide (F), potassium permanganate (P), mercuric chloride (H), sodium dichloroisocyanurate (NaDCC, N), and Domestos® (D). Analysis of co-application frequencies among disinfectants revealed structured associations rather than random usage. The most frequent binary pair was potassium permanganate + mercuric chloride (P|H, n = 19), closely followed by permanganate + fungicide (P|F, n = 19) and permanganate + Domestos® (P|D, n = 16), indicating that permanganate was a core component of multi-agent protocols. The most common ternary association was Domestos® + $HgCl_2$ + permanganate (D|H|P, n = 14), reflecting the recurrence of oxidant-heavy treatments across genotypes.
NaDCC was applied more selectively, co-occurring most often with Domestos® (n = 9) and $HgCl_2$ (n = 3), and never with fungicide (n = 0), suggesting a procedural separation between NaDCC-based and fungicide-based treatments. The high diagonal count for Domestos® (n = 24) also confirms its near-universal presence in disinfection protocols.

Disinfection outcome for *H. macrophylla* "Tivoli" explants under various compound profiles

A total of 146 explants of the "Tivoli" cultivar were subjected to disinfection procedures involving different combinations of five agents (Fig. 1). Overall, only 17 explants remained contamination-free, resulting in a low disinfection success rate of 11.64%, consistent with the cultivar's previously observed recalcitrance. This dataset reflects both primary inoculation and resterilization attempts.

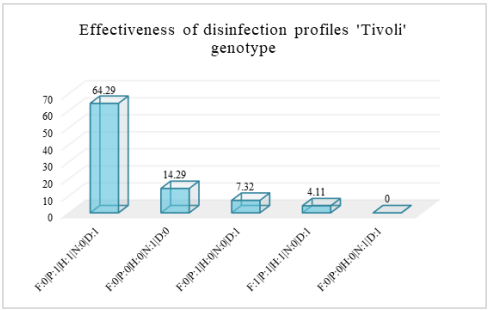

Figure 2. Effectiveness of disinfection profiles "Tivoli" genotype

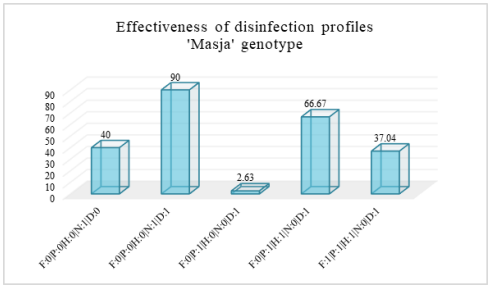

Figure 3. Effectiveness of disinfection profiles "Masja" genotype

Five distinct disinfection profiles were applied with equal frequency (n = 2 for each). The most effective combination was P + H + D (without F or N), which achieved a 64.29% clean rate. (Fig.2) In contrast, the complete profile F + P + H + D (omitting N) resulted in only 4.11% success, and combinations based solely on N, either with or without D, proved entirely ineffective (0 - 14.29%). These findings indicate a strong dependence on mercuric chloride and permanganate for successful decontamination of "Tivoli" explants, with sodium dichloroisocyanurate providing no benefit when applied alone or with Domestos®. The data suggest that "Tivoli" requires aggressive chemical treatment to achieve acceptable decontamination, with mild or NaDCC-based protocols resulting in persistent microbial presence.

Disinfection outcome for *H. macrophylla* "Masja" explants under various compound profiles

A total of 148 explants of the "Masja" cultivar were processed through primary inoculation and repeated re-sterilization steps. Eleven disinfection treatments were categorized according to the combinations of five chemical agents and the most effective was the combination N + D (without F, P or H), which resulted in a 90.00% clean rate across 10 explants (Fig.3). In contrast, the poorest outcome was associated with the P + D profile, which yielded a 2.63% success rate (1 out of 38 explants). The P + H + D profile demonstrated intermediate efficacy, reaching 66.67%, while the complete combination F + P + H + D reached 37.04%, confirming that the inclusion of NaDCC was not essential to achieving high efficacy in this cultivar.

Disinfection outcome for *H. macrophylla* "Love' explants under various compound profiles

The overall success rate of disinfection was 31.07% for "Love" cultivar and eleven treatment events were assigned to five disinfection profiles, with the highest clean rate was recorded for the N + D profile (without F, P, or H), reaching 87.5% (7 out of 8 explants). The lowest performance was seen with the complete profile F + P + H + D, which yielded only 28.00% clean material. Profiles including P + H + D or P + D alone resulted in intermediate rates of 23.81% and 26.67%, respectively, indicating reduced efficacy in the absence of NaDCC (Fig. 4). Treatments based solely on N, without D, F, or oxidants, were ineffective (22.22%).

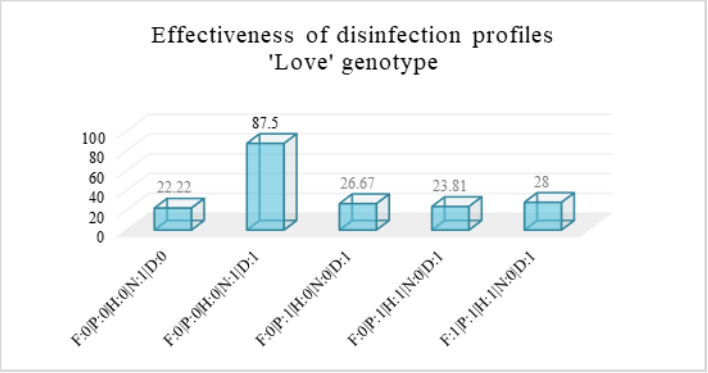

Figure. 4 Effectiveness of disinfection profiles "Love" genotype

For the "Love" genotype, successful disinfection is highly dependent on the presence of both NaDCC and Domestos®, with limited added benefit from the inclusion of additional agents such as permanganate or mercuric chloride. This genotype displays a moderate tolerance to complex chemical mixtures and may benefit from simplified protocols emphasizing chlorine-based oxidants.

Genotype-dependent response to disinfection profiles in *Hydrangea macrophylla*

A detailed comparative analysis of the applied disinfection protocols revealed substantial overlap in the treatment profiles among the three *Hydrangea macrophylla*. Across all inoculation and resterilization events, 13 distinct treatment combinations were identified based on the presence or absence of the five agents (Fig. 5. The most frequent protocol applied to "Love" included potassium permanganate, $HgCl_2$, and Domestos®, but omitted NaDCC and fungicide (36% of events), while 'Masja' was most often treated using a full combination of permanganate, $HgCl_2$, Domestos®, and occasionally fungicide (notably Dianthe® at 0.2 g L^{-1}). For "Tivoli", the treatments were more uniform, with Domestos® and NaDCC used in 75% and 50% of cases, respectively, although fungicide use remained low (16.7%). Statistical evaluation using chi-square tests ($p > 0.79$ for all comparisons) confirmed that there were no significant differences in the frequency of any individual treatment between the three genotypes

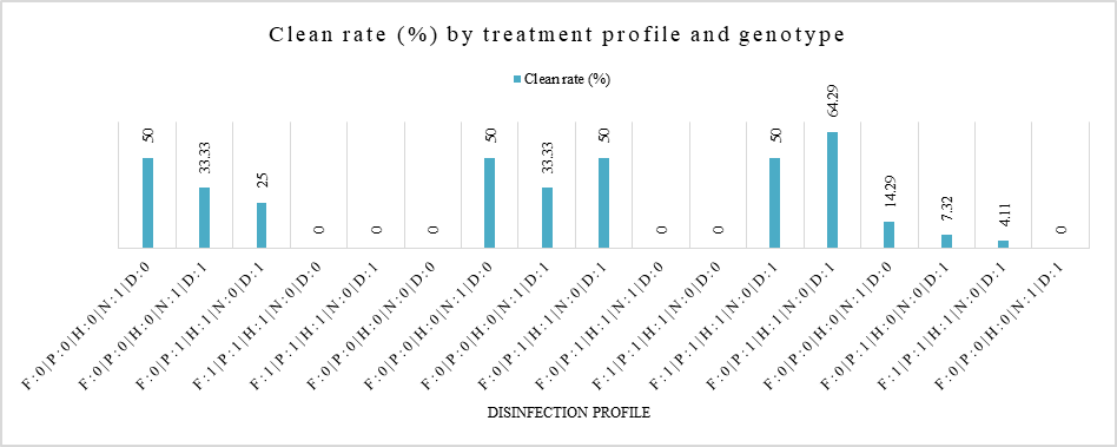

Fig.5 Comparative efficacy of disinfection profiles among Hydrangea macrophylla genotype

A Chi-square analysis applied to the full disinfectant profile (F + P + H + D) revealed a statistically significant difference in disinfection success among "Tivoli", "Masja", and "Love" ($\chi^2 = 6.67$, $p = 0.0159$), with "Tivoli" performing markedly poorly under complex chemical regimes compared to the other two cultivars, which consistently exceeded 30% clean rates. In contrast, simpler profiles such as NaDCC + Domestos® or permanganate-based combinations produced uniform efficacy across genotypes without statistically significant differences. These results suggest that "Tivoli" exhibits lower tolerance to multi-oxidant treatments, whereas "Masja" and "Love" respond more favorably to milder, chlorine-based disinfection protocols.

Literature in plant tissue culture supports our findings: standard sterilization methods often vary in effectiveness depending on genotype, explant type, and oxidative damage thresholds and recalcitrant genotypes (Hesami et al., 2025, Boccon-Gibod et al., 2000). For *Hydrangea*, early work by Cassells (1997); Douglas (1986), Holdgate & Zandvoort (1997), reported contamination rates up to 69% in meristem-tip explants, emphasizing that genotype and endophytic burden affect sterilization outcomes. More broadly, studies with other ornamental species highlight the trade-off between disinfection efficacy and cytotoxicity: mercuric chloride ($HgCl_2$) often achieves near 100% decontamination at specific exposures, but longer treatments significantly reduce explant survival, whereas NaOCl or NaDCC to a lesser extent provide moderate sterilization with higher viability (Thomas & Heuser, 1987). Our comparative dataset is therefore aligned with prior evidence showing that genotype-specific sensitivity to sterilant composition and exposure is a critical determinant of in vitro success.

CONCLUSION

The three *Hydrangea macrophylla* cultivars displayed distinct responses to disinfection protocols. "Masja" showed the highest overall survival when treated with a complite combination of potassium permanganate, mercuric chloride, Domestos®, and fungicide. "Love" responded best to milder treatments based on

NaDCC and Domestos®, while "Tivoli" achieved the highest decontamination rate when treated with the oxidant combination of permanganate and $HgCl_2$, without NaDCC or fungicide. These findings may support the importance of genotype-specific optimization in disinfection protocols for *in vitro* culture of *Hydrangea spp*.

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