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## Recent advances in understanding and controlling hyperhydricity during plant micropropagation

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### Abstract

Hyperhydricity (historically termed vitrification) remains one of the most frequently reported physiological disorders in plant tissue culture, widely described as a major recurring cause of shoot-quality loss, reduced multiplication rate, and poor post-transplant survival across more than 200 species. This review synthesizes the literature of the last eight years (2018-2026) on the physiological, biochemical, epigenetic, and structural basis of hyperhydricity, and critically evaluates recently reported strategies for its prevention and reversion. Water saturation of the apoplast, driven by imbalanced water potential between explant and gelled or liquid medium, is now recognized as the proximate structural cause, while an ethylene-reactive oxygen species (ROS) signaling axis, disrupted nitrogen and mineral metabolism, aquaporin phosphorylation, cell-wall hypolignification, and DNA hypomethylation of ethylene-pathway genes have been identified as interconnected downstream and upstream drivers. Transcriptomic and proteomic studies in *Arabidopsis*, flax, *Dendrobium*, oil palm, and *Vaccinium* species have refined this mechanistic picture. On the control side, this review evaluates medium and gelling-agent optimization, alternative aromatic cytokinins (meta-topolin derivatives), silver nitrate and biogenic silver nanoparticles, polyamines, silicon supplementation, ventilation and forced gas-exchange systems, temporary immersion bioreactors, and physical interventions such as bottom cooling. Emerging spectral-imaging and machine-learning tools for early, non-destructive detection of hyperhydricity are also discussed as a promising direction for precision micropropagation. The review concludes that hyperhydricity control must remain species- and even cultivar-specific, and it proposes a research agenda integrating multi-omics, computer vision, and bioreactor engineering to achieve predictive, rather than reactive, management of this disorder in commercial micropropagation; its scope, search strategy, and limitations are stated explicitly in Sections 1.1 and 8.

**Keywords:** Hyperhydricity, vitrification, micropropagation, tissue culture, oxidative stress, ethylene, silver nanoparticles, temporary immersion system, machine learning

### 1. Introduction

Micro propagation is a way to multiply plants in a clean environment. This method is very important for the nursery, forestry and ornamental industries. It also helps to conserve plants. Micropropagation lets us make plants that are free of disease and exactly the same. We can make a lot of plants this way which's not possible with other methods. Even though we have been working on micropropagation for a time it is still not, like how plants grow in nature. The containers we use are closed and have much moisture. This makes it hard for the plants to get the air they need. We also add a lot of sugar, salt and other things that help plants grow. These are not natural. Because of this the plants we grow using micropropagation often do not grow properly. They can get a condition called hyperhydricity, which means they get much water and do not develop normally. Micropropagation and the condition of hyperhydricity are problems that we are still trying to fix.

Hyperhydricity. Also known as vitrification in the past a word that is now mostly used for a way of freezing plant material. Is a condition where the leaves and stems look clear and wet like glass. The leaves and stems are hard. May be curled or wrinkled. The outer layer of the leaves is not there or is less the tiny holes on the leaves don't work as they should the parts that hold the plant together are weaker than normal. The plants don't do well when moved from the lab to the real world <sup>[1, 2]</sup>. This problem was first noticed fifty years ago. Since then it has been found in than 200 different types of plants. These include plants that are grown for decoration, fruit and tree plants plants used for medicine and even orchids and

palms. It is often mentioned in papers as a big reason for losing crops in the process of growing plants in labs. However as explained in Section 8 few studies actually measure how much this loss costs, in money <sup>[2, 3]</sup>.

The problem of hyperhydricity has been talked about a lot since the 1980s.. In the last eight years we have learned a lot more about what causes it. This is because of ways of studying hyperhydricity, such as looking at the genes and metabolism of the plants. We have also made progress in growing plants in containers and using new materials to help them grow.. Recently we have started using computers to help us understand what is happening to the plants.

This review looks at all the information we have about hyperhydricity from the last few years from 2018 to 2026. We want to give an up-, to-date picture of hyperhydricity including what it looks like how it happens and what things can make it more or less likely to occur. We also want to talk about ways to detect and control hyperhydricity. Our goal is to help people who are studying hyperhydricity and also to help companies that are growing plants on a scale. We want to help them understand hyperhydricity and find ways to deal with it.

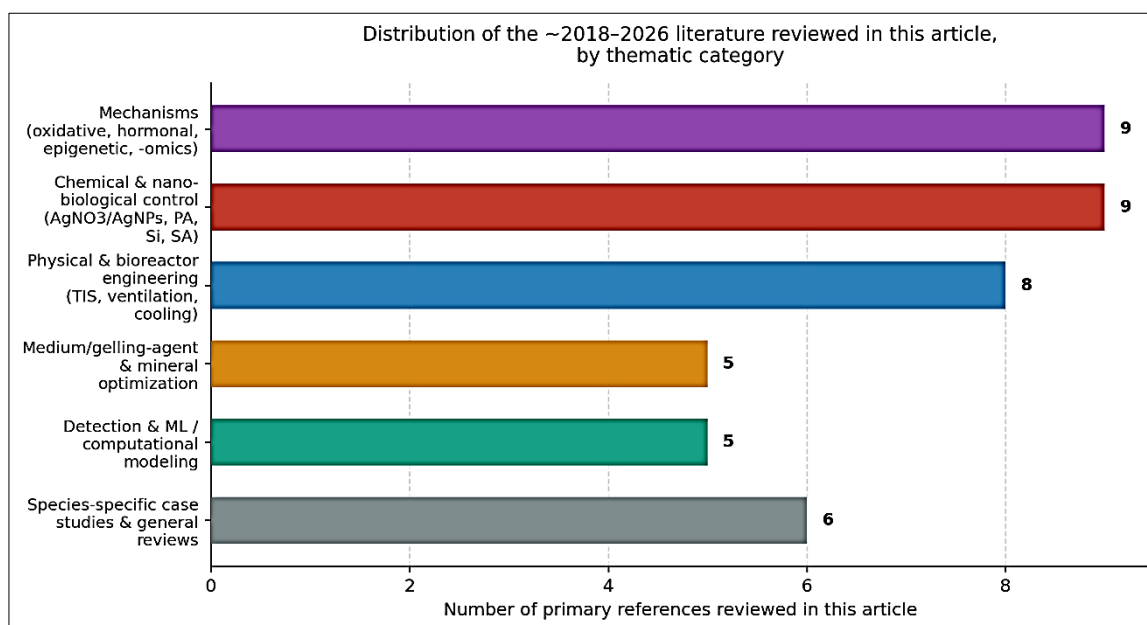
### 1.1 Scope and Literature Search Strategy

This is a structured narrative review rather than a formal systematic review: no pre-registered protocol was used, and study selection, while documented below, was not independently duplicated by a second reviewer. Readers requiring a fully systematic, PRISMA-compliant synthesis (e.g., for a Cochrane-style evidence grading) should treat the quantitative comparisons in this article as illustrative of reported effect directions rather than as a meta-analytic estimate of effect size.

The review targeted primary research articles, reviews, and dissertations addressing hyperhydricity (or its historical synonym vitrification, excluding cryopreservation-related uses of the latter term) in plant tissue culture and

micropropagation, published between January 2018 and July 2026. Sources were identified through structured searches of Consensus, PubMed, and general academic web search using combinations of the terms "hyperhydricity", "vitrification", "micropropagation", and "tissue culture", together with mechanism- and method-specific qualifiers (e.g., "ethylene", "oxidative stress", "silver nanoparticles", "temporary immersion", "machine learning"). Reference lists of major recent reviews were additionally screened to capture studies not directly retrieved by keyword search, and reference lists of already-included studies were checked in turn (snowball sampling) to identify independent replications of key findings in unrelated species and laboratories.

Because this was not done as a -registered systematic review there was no formal PRISMA-style screening count recorded in advance and this review intentionally does not show a reconstructed funnel of search-to-inclusion numbers since doing that after the fact would suggest a level of accuracy that the original process did not provide. In terms many more candidate records were looked at than were finally included, with exclusions mostly because they were not related to the topic (for example nanoparticle or tissue-culture studies that did not specifically look at hyperhydricity) because the publication date was not between 2018 and 2026 or because there was not enough methodological detail to get a useful finding from them. Of the 43 sources that are included in this review digital object identifiers (DOIs) were checked separately against publisher or CrossRef-indexed information for 26 of them (~60%); the other 17. Mostly theses, conference papers and a few journal or, in-press articles. Are only cited by journal, volume and page numbers and the reader should check the bibliographic details again before using them in any formal citation. Figure 1 shows the main themes of the literature included in this review.



**Fig 1:** Distribution, by thematic category, of the primary literature (2018-2026) synthesized in this review

## 2. Definition, Terminology and Diagnostic Features

Hyperhydricity is a condition that happens when shoots or somatic embryos grown in a laboratory setting develop a problem. This problem is caused by much water building up

in the spaces between cells and in the apoplast. The condition causes the cells to have problems with their structure and function. It leads to lignification, thinner cell walls issues with the way chloroplasts develop and problems

with how the stomata work [1, 2]. On the outside the shoots look clear, swollen and shiny like glass. The leaves might be curled, thick and break easily. When looking closely under a microscope the spaces between cells are bigger. Have collapsed. The layers of the leaf tissue are not clearly separated. The parts of the plant that carry water and nutrients are not fully developed. The stomata, which are openings, on the leaves might be open not shaped properly or not work at all even when they should close [2, 4].

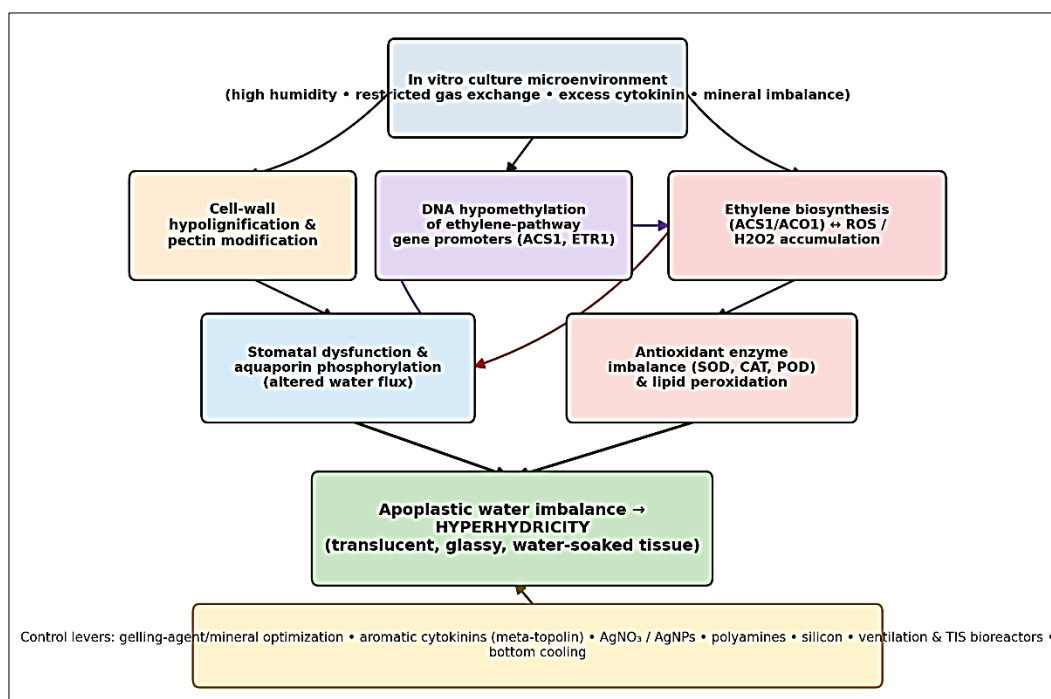
In the year 2025 a study that used a scanning electron microscopy and magnetic resonance to look at blueberry hybrids that were not normal they found some things that were different. The blueberry hybrids had spaces between the cells and the vessels that carry water and food were not working right. The connections between the vessels were weak. The parts that help the plant breathe were closed.. The study also found that these blueberry hybrids had more of some helpful chemicals like catechin than normal plants. This is interesting because it seems that when the plant is under stress it can make more of these chemicals.

Other people have seen the things in other plants like *Arabidopsis* and apple. They have cell walls and their membranes are damaged. They also have fewer of the parts

that help the cell work and their vessels are collapsed. This is similar to what was seen in the blueberry hybrids. The scanning electron microscopy and nuclear magnetic resonance study of blueberry hybrids found these things. It is like what was seen in other plants. The blueberry hybrids had spaces, between the cells and the vessels that carry water and food were not working right just like in other plants.

### 3. Underlying Physiological and Molecular Mechanisms

In *Arabidopsis thaliana* *Dianthus chinensis* and several other species discussed below hyperhydricity has been connected to a network of processes that are linked together than a single cause. The conditions inside the culture vessel affect cell-wall chemistry, the status of DNA methylation and signals involving ethylene and ROS. These factors all play a role in the problems with stomata. The imbalance of antioxidants that happen along with the water imbalance in the space, between plant cells that causes hyperhydricity (Figure 2). How much this network, which is mostly based on a group of well-studied species can be applied to other cases is looked at later in this section.



**Fig 2:** Conceptual schematic of the principal interconnected mechanisms implicated in hyperhydricity onset, synthesized from the reviewed 2018-2026 literature [1, 2, 4, 6, 7, 8, 9].

#### 3.1 Water Imbalance in the Apoplast

The main structural reason for hyperhydricity is now mostly known to be an imbalance between water coming in and water leaving at the tissue level. When the water taken up from the gelled or liquid medium is more than what's lost through transpiration it causes problems. This happens because of humidity around the vessels problems with the stomata and cell walls that are not strong enough and let water pass through easily. Water builds up in the apoplast and between the cells. This stops gas movement and the exchange of gases needed for photosynthesis. It also creates an area, with little oxygen, which makes the problem worse [1, 2].

#### 3.2 Ethylene Signaling and Oxidative Stress

There is a lot of evidence that says ethylene signaling is a reason for hyperhydricity. When the genes that help make ethylene like ACS1 and ACO1 are very active it is often linked to hyperhydricity in China plants. If you add a chemical called ethephon to these plants it can cause hyperhydricity.. If you use chemicals like silver nitrate or cobalt chloride it can stop hyperhydricity and reduce the activity of the ACS1 and ACO1 genes.

The silver ions seem to work by blocking the plants ability to sense ethylene, which helps the plants stomata work properly and reduces the amount of water that accumulates outside the plant cells. Ethylene signaling is very important,

in this process. When it is disrupted it can lead to hyperhydricity.

The ethylene signaling pathway is complex. It involves many different genes and chemicals including ACS1 and ACO1.

The model that is centered around ethylene is closely connected to something called oxidative stress. When tissues get much water they always have high levels of hydrogen peroxide and superoxide. They also have malondialdehyde, which shows that lipids are being damaged and the enzymes that prevent damage from oxygen like superoxide dismutase and catalase do not work properly. Ethylene and something called ROS signaling seem to work to affect the way stomatal guard cells and aquaporin work. This connection helps us understand how ethylene signaling and water relations are linked. The ethylene model and ROS signaling appear to converge on guard-cell function and on aquaporin activity, which is important, for the water-relations phenotype of ethylene and the model that is centered around ethylene.

### 3.3 Epigenetic Regulation

A study in 2022 on *Arabidopsis thaliana* used a technique to look at the entire genome. This study found out that hyperhydricity is related to epigenetics. The seedlings that were hyperhydric had changes in their genome in the parts that control the genes ACS1 and ETR1. These genes were more active. This was connected to the seedlings having more ethylene.

The hyperhydric seedlings also had problems with their stomata, which're like the lungs of the plant and they took up more water.. When the seedlings were treated with silver nitrate the genes ACS1 and ETR1 were less active and the seedlings had less ethylene and took up less water.

This study shows that changes in the DNA the removal of methyl groups play a big role in the development of hyperhydricity which is a water-relations disorder caused by ethylene. The epigenetic state is, like a switch that controls how the genes work. This switch is connected to the ethylene-driven disorder.

### 3.4 Cell Wall Modification and Lignification

Hypolignification of the cell wall. Less lignin being added in epidermal tissues. Is a common feature of hyperhydric explants and is believed to make the walls weaker stop the control of water loss through transpiration and make tissues more likely to get flooded in the apoplastic space. Doctoral research on *Arabidopsis* and *Limonium sinuatum* found hypolignification to be a cause and showed that adding calcium from outside the plant along, with the lignin building block p-coumaric acid and the stomatal opener 5-aminolevulinic acid (ALA) helped reduce or delay hyperhydricity by encouraging the making of lignin and changing the leaf pectin levels <sup>[12]</sup>. These results link the chemistry of the cell wall directly to the water balance and the structure problems that were talked about earlier.

### 3.5 What We Learned From Studying Genes and Hormones:

We now know a lot more about what's going on with plants at a molecular level. Scientists looked at the genes and hormones of flax plants that were not growing normally and found 2,700 genes that were not working the way they should. These genes are important for things like making chemicals and helping the plant respond to signals. They also found that most of the hormones in these plants were not at levels except for one type called cytokinins. This suggests that the problem is not just with one thing but with things in the plant.

In another study scientists looked at a type of orchid. Found that adding silicon helped the plant grow normally again. They used something called analysis to see how the silicon was affecting the genes in the plant. Scientists also studied oil palm plants. Found that some genes were working too much in plants that were not growing normally. These genes are important for things like responding to stress and making energy. They are also important for helping the plant defend itself and for sending signals.

All of these studies together tell us that while some things, like ethylene and oxidative stress are still important the problem is really about genes and systems in the plant not working together properly.

**Table 1:** summarizes the things we learned and the plants that were used in the studies along, with where to find more information.

| Mechanistic axis                              | Representative evidence                                                                                      | Species studied                                         | Ref(s)               |
|-----------------------------------------------|--------------------------------------------------------------------------------------------------------------|---------------------------------------------------------|----------------------|
| Apoplastic water imbalance                    | Enlarged intercellular spaces; flooded apoplast; malfunctioning stomata                                      | Blueberry, <i>Arabidopsis</i> , apple, many others      | [1], [2], [4]        |
| Ethylene overproduction (ACS1/ACO1)           | Ethephon reproduces HH; AgNO <sub>3</sub> /CoCl <sub>2</sub> reverse it and down-regulate ACS1/ACO1          | <i>Dianthus chinensis</i> , <i>Lycium ruthenicum</i>    | [6], [10], [11]      |
| Oxidative stress / ROS imbalance              | Elevated H <sub>2</sub> O <sub>2</sub> , O <sub>2</sub> <sup>•-</sup> , MDA; altered SOD/CAT/POD/GR activity | <i>Dianthus</i> , <i>Gypsophila</i> , banana, blueberry | [1], [4], [14], [15] |
| DNA hypomethylation of ethylene genes         | Genome-wide CHH hypomethylation; ACS1/ETR1 promoter demethylation                                            | <i>Arabidopsis thaliana</i>                             | [7]                  |
| Cell-wall hypolignification                   | Reduced lignin deposition; altered pectin; thin, weak cell walls                                             | <i>Arabidopsis</i> , <i>Limonium sinuatum</i>           | [1], [12]            |
| Broad hormonal / transcriptomic dysregulation | ~2,700 DEGs; reduced hormone levels except cytokinins; MAPK & phenylpropanoid pathways enriched              | <i>Linum usitatissimum</i> (flax)                       | [8]                  |
| Systemic stress-gene activation               | 17/19 candidate genes up-regulated (ethylene synthesis, energy metabolism, defense)                          | Oil palm ( <i>Elaeis guineensis</i> ) somatic embryos   | [9]                  |

We need to be careful. If we look at Table 1 we can see that some of the detailed findings like the DNA-demethylation switch and the data on ethylene and ROS in Table 2 are based on a small number of species mainly *Arabidopsis thaliana* and *Dianthus chinensis*, that have been studied in detail by a few research groups. We do not know if the

specific molecular details, like the hypomethylation of CHH at the ACS1 and ETR1 promoters are the same in all the species that are affected by hyperhydricity. It is possible that these details are unique to the species that have been studied or that they are one example of how different species can achieve the same physiological result. Because we have not

done studies of many species using the same methods we cannot say for sure. So when we look at the model in Figure 2 we should think of it as the explanation we have so far rather than a proven pathway that applies to all species. The DNA-demethylation switch and the model in Figure 2 are based on the data, from *Arabidopsis thaliana* and *Dianthus chinensis* and more research is needed to see if they apply to species.

#### 4. Culture-Based Factors Influencing Hyperhydricity Incidence

##### 4.1 Gelling Agent Type and Concentration

The type of gelling agent and how much of it is used have an impact on hyperhydricity incidence. This is because they affect the water potential in the medium and how easy it is for roots and shoots to take up water. For example in *Salvia santolinifolia* when the amount of agar was reduced from 0.8 percent to 0.4 percent hyperhydricity increased from 39 percent to 72 percent.

The type and amount of cytokinin and the levels of nitrogen and calcium salts also interacted with the effects of the gelling agent. This shows that to control hyperhydricity many factors need to be considered not one. Similar results were seen in raspberry plants, where reducing the amount of agar from 8 grams per liter to 6.5 grams per liter increased the rate at which plants were produced but also caused hyperhydricity in the shoots. This is a trade-off between how well plants are produced and their quality.

Gellan gum is more likely to cause hyperhydricity than agar when used at the concentrations. This is because the structure of the gel and how it releases water are different.

Recent studies using sensors have shown that the humidity and water availability vary within culture vessels and are different for agar and gellan gum. This helps explain why hyperhydricity can be worse in some parts of a culture tube, than others.

##### 4.2 Mineral Nutrition

Excess ammonium relative to nitrate, and imbalances in calcium, potassium, and other macronutrients, are well-established contributors to hyperhydricity. Machine-learning-assisted modeling of *Actinidia arguta* culture media identified nitrogen, potassium, phosphorus, magnesium, sulfur, and several micronutrients as the key ions governing hyperhydricity risk alongside shoot quality and callus formation, and an optimized medium derived from this model significantly reduced hyperhydricity while improving shoot quality<sup>[18]</sup>. In *Gypsophila paniculata*, fortifying the medium with full-strength potassium and ammonium nitrate combined with the growth retardant paclobutrazol and a higher agar concentration (9 g L<sup>-1</sup>) successfully reverted hyperhydric shoots and restored high-quality multiplication and rooting, accompanied by enhanced peroxidase, catalase, and polyphenol oxidase activity<sup>[14]</sup>.

##### 4.3 Cytokinin Type and Concentration

Cytokinin identity and dose remain among the strongest and most consistently reported determinants of hyperhydricity. 6-Benzylaminopurine (BA), the most widely used cytokinin in micropropagation, is frequently implicated in dose-dependent induction of hyperhydricity, whereas aromatic topolin-type cytokinins meta-topolin (mT), meta-topolin riboside (mTR), and meta-methoxy topolin riboside (MemTR) have repeatedly been shown to sharply reduce or

eliminate hyperhydricity relative to BA while maintaining or improving shoot multiplication and quality. In pear (*Pyrus communis*), replacing BA with 5 µM MemTR or mTR in a SETIS™ temporary immersion bioreactor completely inhibited hyperhydric shoot formation while producing the highest shoot numbers and leaf area of the treatments tested<sup>[19]</sup>.

##### 4.4 Vessel Ventilation and Gas Exchange

Restricted gas exchange, which elevates internal vessel humidity and CO<sub>2</sub>/ethylene accumulation, is one of the most direct environmental triggers of hyperhydricity, and increasing ventilation is correspondingly one of the most broadly effective countermeasures. In the Andean root crop *Arracacia xanthorrhiza*, forced ventilation at 81.3 air exchanges per day reduced hyperhydricity to 8.3%, compared with 65.8% in sealed vessels, while combining ventilation with RITA® temporary immersion increased shoot multiplication by 48% over semi-solid medium and improved leaf expansion and shoot morphology<sup>[20]</sup>. Similarly, in potato (*Solanum tuberosum*), vessel closures with three ventilation holes produced the best plantlet quality among four tested closure types, and silver nitrate supplementation was the only chemical additive tested that reliably produced high-quality, non-hyperhydric plantlets under tightly sealed conditions<sup>[21]</sup>.

##### 4.5 Light, Temperature, and Vessel Microclimate

Light intensity and quality, and culture-room temperature, further modulate hyperhydricity risk, generally through their effects on transpiration and stomatal behavior, although the studies reviewed here characterize this relationship mainly qualitatively rather than through systematic dose-response data, and a general rule linking specific photon flux densities to stomatal function cannot be extracted with confidence from the present literature set. A notable 2025 innovation is the use of localized bottom cooling (creating an approximately 2 °C temperature differential beneath culture vessels) during the culture-initiation stage of *Cannabis sativa* micropropagation, which significantly improved survival, chlorophyll content, chlorophyll fluorescence ratios, and cuticle/stomatal function relative to standard shelving, and markedly reduced the proportion of translucent, brittle, hyperhydric explants<sup>[22]</sup>. This physical, low-cost intervention illustrates that microclimate engineering at the level of the culture vessel rather than medium chemistry alone can meaningfully reduce hyperhydricity incidence, particularly during the vulnerable culture-initiation phase; however, because it has so far been reported in a single species, its generality across taxa with different cuticle biology remains untested.

##### 4.6 Controversies and Conflicting Evidence

Not all factors reviewed above act across species and a critical synthesis should make these disagreements clear instead of showing only the usual result. Three examples show this. First while gellan gum is often seen as likely to cause hyperhydricity than agar when used in the same amounts this ranking is not always true. Because the gel structure, ash or impurity levels and the amount of water available vary between gellan and agar products at least one review of the original studies points out that some studies found no improvement in hyperhydricity. Or even worse problems. When gellan gum was used instead of agar, which

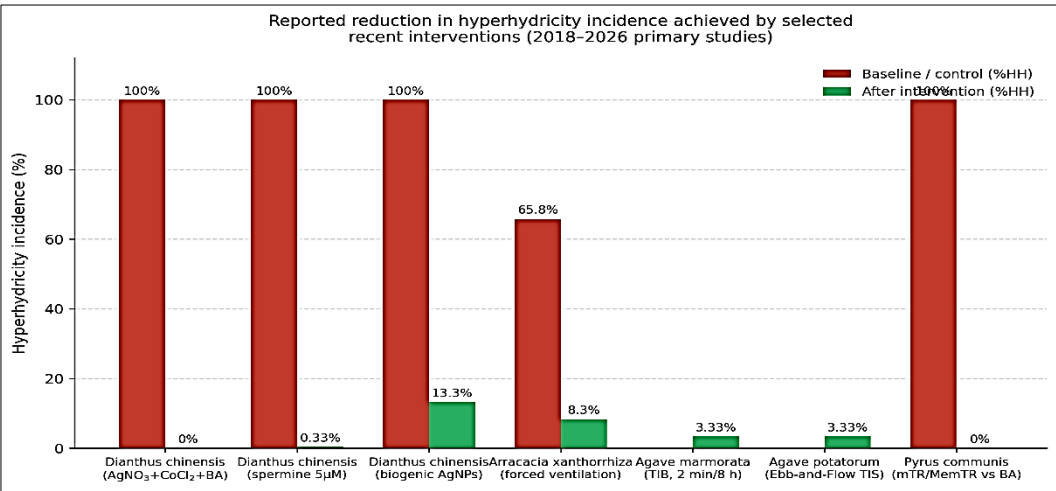
is the opposite of what is usually reported [1]. So the general idea should be seen as a tendency, not a rule. Second how cytokinins work depends a lot on the plant type. While meta-topolin compounds completely stopped hyperhydricity compared to BA in pear [19] the studies here do not show that this benefit works the same in other plants. BA is still the cytokinin that people use most for growing shoots in methods where hyperhydricity is not a problem. Third and clearly shown in the studies reviewed here adding silver nanoparticles helped improve the quality of shoots and reduced hyperhydricity in temporary immersion culture of apricot. But the same nanoparticles were reported to be harmful in semisolid agar for the varieties with very different levels of silver found in the plant tissue, in each system [23]. This is a clear example that the effect of an action can change completely depending on the culture system. These examples show that you should not assume

any one solution will work for all plants without checking at the species or even the variety level.

**5. Recent Advances in Control and Reversion Strategies**  
Table 2 and Figure 3 show what we found out about some chemical, biological and engineering-based interventions. We only included studies that gave us numbers for before and after hyperhydricity incidence. This is the rule we used in Section 4 when we left out the calcium-nitrate banana study in Section 5.5 because it only told us about rooting percentage, not hyperhydricity incidence. Some other things we tried like using topolin and temporary immersion for *Pyrus communis* and temporary immersion bioreactors, for *Agave* spp. And apple, also helped a lot with hyperhydricity but the studies only gave us general ideas, not exact numbers so we can only talk about them in the text and show them in Table 3.

**Table 2:** Quantified before/after outcomes of hyperhydricity-control interventions reported in 2018-2026 primary studies (both baseline and treated values independently quantified in the source publication)

| Species                       | Intervention                           | Baseline HH           | HH after treatment      | Ref  |
|-------------------------------|----------------------------------------|-----------------------|-------------------------|------|
| <i>Dianthus chinensis</i>     | AgNO3 (10 µM) + CoCl2 (5 µM) + BA      | 100%                  | 0% (complete reversion) | [10] |
| <i>Dianthus chinensis</i>     | Spermine, 5 µM                         | 100%                  | 0.33%                   | [6]  |
| <i>Dianthus chinensis</i>     | Biogenic AgNPs, 100 µg L <sup>-1</sup> | 100%                  | 13.3%                   | [24] |
| <i>Arracacia xanthorrhiza</i> | Forced ventilation, 81.3 exchanges/day | 65.8% (sealed vessel) | 8.3%                    | [20] |



**Fig 3:** Reported hyperhydricity incidence before and after selected interventions, drawn from the primary studies summarized in Table 2. Each bar pair is a single-study point estimate; cross-study comparisons should be interpreted qualitatively, not as a formal meta-analysis.

The *Dianthus chinensis* results in Table 2 come from a single research group across companion publications [6, 10, 24]; the exact reversion percentages shown should therefore be read as single-laboratory estimates. Section 5.1 discusses independent evidence bearing on the generality of the underlying AgNO3/ethylene mechanism.

**5. Silver Nitrate and Silver Nanoparticles**

Silver nitrate (AgNO3) a substance that stops ethylene from being sensed is one of the often mentioned chemical things used to control hyperhydricity. This effect is not seen by one group of researchers or one type of plant. In addition to the work on *Dianthus chinensis* that is discussed below a study from 2023 on snapdragon (*Antirrhinum majus* cv. Maryland Apple Blossom). A lab and a different family of plants. Also found that AgNO3 lowered ethylene-related gene activity and the build-up of reactive oxygen species reduced the space between leaf cells and increased the number of working stomata. This allowed the hyperhydric shoots to

become plants when moved [25]. More recent studies have also taken the AgNO3 method further by using biogenic and man-made silver nanoparticles (AgNPs). These seem to have the ability to fight ethylene along with effects on how water moves and the level of antioxidants. In *Dianthus chinensis* biogenic AgNPs made from leaf extract brought hyperhydricity down from 100% (without treatment) to 13.3% at 100 µg L<sup>-1</sup>. It also cut the H2O2 level by half and made the ACS1/ACO1 genes less active when the plants returned to normal. ISSR analysis showed that the plants treated with AgNPs stayed genetically stable [24]. Similar results with AgNPs in reducing hyperhydricity and making plants better have been found in lavender. There 20 mg L<sup>-1</sup> of AgNPs stopped hyperhydricity while making the levels of phenolics and flavonoids higher. The antioxidant activity stronger [26]. In *Gaillardia pulchella* AgNPs helped with shoot growth the amount of chlorophyll and carbohydrates and the development of stomata and hairs. It also removed the overly wet look that was seen in the plants without

AgNPs [27]. In strawberries AgNPs made the cleaning of the plant pieces better helped the shoots and roots grow and encouraged the formation of runners while lowering the amount of ethylene in the containers [28]. It is important to note that the benefits of AgNPs depend on the system being used. In apricot AgNPs helped with the amount of product. Lowered the level of hyperhydration when they were used in a temporary immersion liquid system.. They were bad when used in a semisolid agar medium. Also the amount of silver found in the plant tissue was very different, between the two ways of growing the plants [23].

## 5.2 Polyamines

Exogenous polyamines putrescine, spermidine, and spermine have emerged as effective, comparatively low-cost agents for hyperhydricity reversion, acting at least partly through suppression of ethylene biosynthesis gene expression. In *Dianthus chinensis*, 5  $\mu\text{M}$  spermine reduced hyperhydricity incidence to 0.33% (versus 100% in controls) and reduced H<sub>2</sub>O<sub>2</sub> content by half, while 5  $\mu\text{M}$  spermidine maximized the number of healthy reverted shoots; both treatments down-regulated ACS1 and ACO1 relative to hyperhydric controls [6]. Salicylic acid has shown a related, though hormetically dose-dependent, effect in potato: a low dose (1  $\mu\text{mol L}^{-1}$ ) ameliorated the phenotypic and physiological hallmarks of hyperhydricity restoring antioxidant enzyme activities and polyamine content close to normal-shoot levels whereas a higher dose (100  $\mu\text{mol L}^{-1}$ ) was less effective [29].

## 5.3 Silicon Supplementation

Silicon has attracted renewed interest as a hyperhydricity-mitigating medium additive. In lingonberry (*Vaccinium vitis-idaea*), a first-of-its-kind 2026 study combining structural, biochemical, and gene-expression analyses showed that shoots grown in both liquid and semi-solid media developed typical hyperhydric symptoms excessive water accumulation, distorted stomata, trichomes, and vascular tissue and that supplementing the medium with 1.2 mM potassium silicate (K<sub>2</sub>SiO<sub>3</sub>) significantly reduced tissue water content, restored normal stomatal and vascular structure as visualized by SEM, and normalized the

expression of oxidative-stress- and hormone-related genes, indicating that silicon acts by simultaneously alleviating oxidative stress and correcting hormonal dysregulation [30]. Complementary transcriptomic work in *Dendrobium officinale* is beginning to characterize the gene networks underlying silicon-mediated hyperhydricity reversion at the molecular level [13].

## 5.4 Temporary Immersion Bioreactor Systems

Temporary immersion systems (TIS), in which explants are periodically flooded with liquid medium and then drained, have become one of the most important engineering-based approaches to hyperhydricity management because immersion frequency and duration can be tuned to balance nutrient/hormone delivery against the risk of tissue flooding. A systematic bibliographic review of TIS use in tree species found that hyperhydricity was the principal problem reported across the literature, and that it was most often mitigated by increasing forced-air injection, lengthening the interval between immersions while shortening immersion duration, and reducing cytokinin concentration [31]. Consistent with this, reducing immersion frequency to three times per day nearly eliminated hyperhydricity in pear cultured in a SETIS<sup>TM</sup> bioreactor [19], while in *Agave marmorata* and *Agave potatorum*, immersion regimes of 2 minutes every 8-12 hours combined with moderate medium volume produced hyperhydricity rates as low as 3.3% alongside substantially higher shoot multiplication than semi-solid controls [32, 33]. In apple (*Malus domestica*) cultivars 'Golden Delicious' and 'Royal Gala', longer incubation periods (10 weeks rather than 6) in RITA<sup>®</sup> bioreactors decreased hyperhydricity and increased the multiplication coefficient, with final hyperhydricity rates of 5-23% depending on cultivar and explant support method the first report of TIS micropropagation for these two commercial cultivars [34]. A recent review of TIS use across fruit-crop micropropagation likewise highlights reduced hyperhydricity, alongside faster multiplication and improved oxygen supply, as a principal advantage of bioreactor systems, while noting that headspace design, illumination, and in-vessel rooting remain areas for further engineering improvement [35].

**Table 3:** Selected temporary immersion system (TIS) studies reporting hyperhydricity-related outcomes, 2018-2026. Unlike Table 2, these studies report several different primary outcome metrics (hyperhydricity incidence, shoot multiplication, or rooting/ acclimatization success); the "Primary metric" column makes this explicit so that rows are not misread as directly comparable

| Species                       | Bioreactor / regime                                                                    | Primary metric                       | Result                                                                 | Ref  |
|-------------------------------|----------------------------------------------------------------------------------------|--------------------------------------|------------------------------------------------------------------------|------|
| <i>Salix viminalis</i>        | Plantform <sup>TM</sup> /RITA <sup>®</sup> , 1 min 3-6 $\times$ /day + hourly aeration | HH incidence (qualitative)           | No hyperhydricity observed; higher multiplication than semi-solid      | [36] |
| <i>Alnus glutinosa</i>        | Plantform <sup>TM</sup> , regime optimized (not further specified)                     | Rooting/acclimatization%             | Improved propagation; rooting/acclimatization >85%                     | [37] |
| <i>Pyrus communis</i>         | SETIS <sup>TM</sup> , reduced to 3 $\times$ /day                                       | HH incidence (qualitative baseline)  | Hyperhydricity minimized; fully inhibited when combined with mTR/MemTR | [19] |
| <i>Agave marmorata</i>        | TIB, 2 min/8 h, 50 mL medium                                                           | HH incidence (%) + shoots/explant    | 3.33% hyperhydricity; 19.6 shoots/explant                              | [32] |
| <i>Agave potatorum</i>        | Ebb-and-Flow, 2 min/12 h, 50 mL medium                                                 | HH incidence (%) + shoots/explant    | 3.33% hyperhydricity; 29.6 shoots/explant                              | [33] |
| <i>Malus domestica</i>        | RITA <sup>®</sup> /Plantform <sup>TM</sup> , 6 $\times$ /day 60 s, 10-week cycle       | HH incidence (%), cultivar-dependent | 5-23% hyperhydricity depending on cultivar                             | [34] |
| <i>Arracacia xanthorrhiza</i> | RITA <sup>®</sup> + forced ventilation                                                 | Shoot multiplication (%) increase    | 48.2% higher multiplication vs. semi-solid; fewer oxidative symptoms   | [20] |

### 5.5 Species- and Crop-Specific Case Studies

Because hyperhydricity susceptibility and the efficacy of countermeasures are strongly genotype- and species-dependent, several recent single-species studies merit specific mention. In banana (*Musa* 'Grand Naine'), supplementing agar- or gellan-gelled medium with 0.5-1 g L<sup>-1</sup> calcium nitrate achieved 100% rooting of hyperhydric shoots (versus 50% without calcium nitrate), lowered electrolyte leakage, reduced H<sub>2</sub>O<sub>2</sub> and superoxide levels, and ultimately supported 100% survival during acclimatization [15]. In *Cannabis sativa*, a widely used vented-lid, low-agar culture-initiation method combined with the bottom-cooling approach described above has substantially improved shoot quality and survival in cultivars historically prone to hyperhydricity [22]. In oil palm, the first in-depth molecular characterization of hyperhydricity during somatic embryogenesis found that hyperhydric embryos retained lower starch reserves and larger intercellular spaces than normal embryos, but that some hyperhydric embryos showed histochemical features resembling normal tissue a finding potentially explaining spontaneously observed reversion from the hyperhydric to the normal state in this and other species [9]. In potato (*Solanum tuberosum*) a systematic review of *in vitro* seed-tuber production techniques found that hyperhydricity was a problem in meristem culture, micropropagation and microtuber production. Showed that temporary immersion bioreactor systems worked well in 65 studies [38]. Another study using a tissue-culture-based nutrient-control system showed that changing the amounts of macronutrients (N, P, K Ca Mg) in the medium could help set the levels that are too low or too high for hyperhydricity in this species [39]. In the fiber-hemp cultivar *Cannabis sativa* 'Cheungsam' creating a micropropagation method needed testing different types of cytokinins (2iP, BA, meta-topolin) and changing between MS and DKW basal media to stop explant necrosis and hyperhydricity. This showed that in one genus the base-salt formula can matter as much, as the growth regulators for controlling hyperhydricity [40].

### 6. Emerging Detection Technologies: Spectral Imaging and Machine Learning

Something new and interesting is happening with the use of optical sensing and machine learning to find hyperhydricity in plants before it is easy to see. This means we can do something about it sooner.

A study in 2023 looked at how light reflects off the leaves of apple and *Arabidopsis* plants that have hyperhydricity. The study found that hyperhydricity has some signs that can be seen when we look at the light. These signs are at 980, 1150, 1400, 1520, 1780 and 1930 nm. The study used machine learning to tell the difference between plants with hyperhydricity and plants that're normal. It was able to do this 85 percent of the time. This is much better, than a way of doing things that was only correct 63 percent of the time. validated on image data showing that it works for automated camera-based hyperhydricity screening in commercial laboratories. Generally ML and computational modeling tools. Artificial neural networks, fuzzy logic, genetic algorithms and design-of-experiments frameworks. Are being used more and more to optimize mineral and hormonal medium composition. These methods aim to reduce hyperhydricity risk as one of goals. Other goals include shoot number, length and callus formation as shown

for *Actinidia arguta* [18]. Recent surveys of ML uses in plant tissue culture say that these methods can greatly reduce the trial-and-error work needed for protocol optimization. However they also point out that model quality depends on organized and large enough experimental datasets. Also understanding what the models are saying is still a topic of research [42]. A similar way of thinking was used for micropropagation of the endemic lily *Lilium akkusianum*. There ML-based analysis of regeneration data was used with methods to check and improve culture-medium recommendations [43]. Together spectral phenotyping and ML-based medium optimization show a move, from dealing with hyperhydricity after it happens to predicting and preventing it through protocols.

### 7. Research Gaps and Future Perspectives

Despite substantial recent progress, several gaps constrain both mechanistic understanding and practical control of hyperhydricity. First, although the ethylene-ROS-DNA methylation axis is increasingly well characterized in model systems such as *Arabidopsis*, its generality across the taxonomically diverse set of species affected by hyperhydricity remains to be confirmed, and comparative multi-species multi-omics studies combining transcriptomics, proteomics, and targeted hormone and metabolite profiling on identical experimental designs would help distinguish conserved core mechanisms from species-specific modulators. Second, the recent discovery of elevated valuable secondary metabolites (e.g., catechin) in some hyperhydric tissues raises the intriguing possibility of deliberately harnessing controlled hyperhydric or hyperhydricity-adjacent stress states for metabolite production in specific systems, a direction that merits systematic exploration distinct from conventional hyperhydricity-avoidance goals [5]. Third we have seen that spectral imaging and machine learning can detect hyperhydricity on without damaging the plants. However we still need to make these tools a part of the process used in commercial micropropagation. This includes making sensors that're affordable creating standardized training datasets that work for different species and providing real-time decision support. We need to do research in this area because it has a lot of potential.

Fourth bioreactor engineering is another area that has a lot to offer. We can improve the design of immersion systems, such as the shape of the space above the plants the uniformity of the light and the support for roots inside the vessel. We can also improve the way we control the exchange of gases. This can help us rely less on additives like silver compounds. These compounds are expensive. Raise concerns, about regulations and the environment especially when it comes to silver nanoparticles when we are working on a commercial scale. Finally, because hyperhydricity control strategies are frequently reported as effective in one species or even cultivar but ineffective or counterproductive in another as illustrated by the contrasting effects of silver nanoparticles in liquid versus semisolid apricot cultures [23] future work should prioritize mechanistic studies that explain this context-dependency, rather than further accumulating single-species empirical protocols in isolation.

**8. Limitations of This Review:** Several limitations of the present synthesis should be stated explicitly. (i) Search

coverage: this is a structured narrative review, not a pre-registered systematic review (Section 1.1); relevant studies indexed only in non-English-language journals, regional databases, or the grey literature may have been missed, and no formal duplicate-screening or inter-rater reliability check was performed. (ii) Verification depth: bibliographic details for 17 of 43 cited sources (~40%) were not independently cross-checked against a DOI registry within the scope of this review and should be re-verified before onward citation. (iii) Source concentration: several of the most quantitatively striking reversal outcomes (Table 2) originate from a small number of research groups working on a small number of model species (notably *Dianthus chinensis* and the *Vaccinium*/blueberry work of one research group across refs. [2, 5, 30]); this raises the ordinary risk of laboratory- or protocol-specific effects being over-generalized before independent replication. An independent replication of the qualitative AgNO<sub>3</sub>/ethylene mechanism was located in an unrelated species and laboratory (snapdragon [25]), which partly mitigates this concern for the underlying mechanism, but the *Vaccinium*/blueberry literature in particular remains concentrated in one research group and would benefit from independent confirmation. (iv) Quality appraisal: this review does not apply a formal risk-of-bias or study-quality instrument (e.g., sample sizes, replication numbers, and statistical methods were not systematically extracted and compared across studies), so readers should consult primary sources before adopting any single quantitative outcome as a benchmark. (v) Publication bias: as in most applied plant-biotechnology literature, studies reporting a successful intervention are more likely to be published than those reporting a null or negative result, which may inflate the apparent reliability of the control strategies discussed in Section 5. (vi) Economic claims: hyperhydricity is widely described qualitatively as economically costly to commercial micropropagation, but the primary literature surveyed here rarely reports standardized economic loss figures (e.g., cost per discarded plantlet, percentage of production runs affected industry-wide); this review therefore avoids quantifying economic impact and flags this as a genuine gap rather than an oversight of omission.

## 9. Conclusion

Hyperhydricity remains a complex, multifactorial disorder rooted in apoplastic water imbalance but driven by an interconnected network of ethylene overproduction, oxidative stress, epigenetic regulation, aquaporin function, and cell-wall biochemistry. Research from 2018 to 2026 has substantially deepened mechanistic understanding through transcriptomic, epigenomic, and ultrastructural studies while simultaneously expanding the practical toolkit available to practitioners including alternative aromatic cytokinins, silver nanoparticles, polyamines, silicon supplementation, optimized gelling-agent and mineral formulations, temporary immersion bioreactors, and physical interventions such as bottom cooling. No single intervention is universally effective; rather, successful hyperhydricity management increasingly depends on combining chemical, physical, and genotype-specific optimization, supported where possible by emerging spectral-imaging and machine-learning tools for early detection and predictive medium design. As detailed in Section 8 this synthesis should be read as a structured but non-exhaustive narrative account; continued integration of mechanistic and applied research, together with independent

replication of the most striking single-laboratory findings, will be essential to translating the substantial recent progress summarized here into reliable, transferable protocols for commercial plant micropropagation.

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