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Review

Effect of media components on hyperhydricity in horticultural crops: A review

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Abstract Tissue culture of horticultural plants under sterile conditions results in numerous genetically uniform and virus-free plants; hence, the technique is widely used. However, *in vitro* culture increases the risk of hyperhydricity, thereby negatively impacting growth quality and causing substantial plant material loss. Hyperhydricity is a physiological, anatomical, and metabolic disturbance caused by various stresses and affects several plant tissues in a highly intricate manner. Hyperhydricity symptoms vary and its occurrence depends on the species or cultivar. This review considers the main physiological, anatomical, biochemical, and molecular symptoms in several horticultural plants. The primary determinants of plant hyperhydricity levels *in vitro* are media components, such as gelling agents and plant growth regulators. Considering these factors, incorporating external additives in media is a viable strategy to tackle hyperhydricity. Nevertheless, not all techniques are universally effective in alleviating hyperhydricity symptoms. Therefore, a thorough examination of hyperhydricity is necessary, and efforts to mitigate hyperhydricity should be complete, intricate, and species-specific.

Keywords Exogenous additives, Gelling agent, Horticultural crops, Hyperhydricity, *In vitro* culture, Media components, Micropropagation, Plant growth regulators

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Introduction

Horticulture is the art and science art of growing vegetables, fruits, ornamental flowers and landscaping for food, medicinal purposes and for aesthetic satisfaction. It is crucial for ensuring worldwide food and nutritional security. Horticulture deals with high-value crops which are intensively cultivated with high infusion of capital in terms of production inputs, labor and technology per land area. Horticultural products have become an essential part of rural economies in many countries, and the increasing global demand for flowers and fruits has turned horticulture into a significant high-technology commercial trade. The propagation of horticultural plants through tissue culture in sterile condition has been widely utilized as it can produce large number of genetically uniform plants regardless of the season and virus-free as well as disease-free plants. However, hyperhydricity (HH) will likely to occur during *in vitro* culture and can cause an impact on the production quality of horticultural crops especially flowers, fruits and non-woody plants.

HH is a phenomenon that occurs in micropropagated shoots caused by the growth and culture conditions which has substantial influence on the survival and quality of the micropropagated plants. HH is a physiological and morphological disorder that can be caused by many stressful conditions and affects multiple components in a highly intricate manner. This disorder affected the physiological, anatomical structures, biochemical and molecular level of crops. Plants suffering with HH have glassy-looking leaves that are translucent, elongated, brittle, wrinkled or curled and irregular in shape. The symptoms of HH can vary and the prevalence of HH is determined by the species or even the specific cultivar. For instance, in certain horticultural crops, signs of HH will only manifest after subculture, while in others, they will appear at the beginning of culture. Some of hyperhydric leaves also showed an accumulation of anthocyanin. Hyperhydric plants demonstrate diminished viability when transplanted to the greenhouse, most likely attributable to the atypical morphology of their leaves. Severe HH causes a reduction in the plant growth and can lead to the deterioration and death of plant tissue, especially when the upper buds are affected.

According to Liu et al. (2017), media components include of basal medium, PGRs and gelling agents. Razdan (2003) stated that water, macronutrients, micronutrients, plant growth regulators (PGRs), vitamins, and sugar in the growth media provide 95% of the nutrients and energy required by plants *in vitro.* The development of HH was significantly influenced by the presence of media components, such as phytohormones and solidifying agents, as well as the preservation of high relative humidity within the culture containers (Kemat et al. 2023). However, various plants have distinct demands for the growth media and conditions within culture containers. The diverse reactions of plantlets to different media and microenvironments contribute to a plethora of factors that impact HH. In this review, we aim to illustrate 1) changes in physiological, anatomical, biochemical and molecular of several hyperhydric horticultural crops which affected by media components and 2) possibilities of amelioration the HH by application of exogenous additives.

Physiological Effects of Media Components

Solidifying gelling agent

The physical and chemical properties of the media have a significant impact on plant development. Gelling agents are employed in plant tissue culture to impart semisolid consistency to otherwise liquid nutrient. There are two types of solidifying gelling agents that are commonly used in plant tissue culture which are agar and gellan gum. Agar is a polysaccharide extracted from seaweeds meanwhile gellan gum is a bacterial polysaccharide. Gellan gum is widely used as it produces a high transparent gel which can provide better observation of root growth compared to the conventional agar gel. Besides using solidifying agents, liquid media is often used in large scale industry to produce huge number of plants with reduced cost.

The types of gelling agents employed contribute to the response of the plants to HH. Badr-Elden et al. (2012) observed that the use of gelrite as solidifying agent caused a significantly lower of shoots multiplications and increased of HH symptoms in watermelon culture compared to media with agar as solidifying agent. Similarly, severe HH symptoms was found in Spiral aloe culture (*Aloe polyphylla*) on gelrite media compared to agar media (Ivanova and Van Staden 2011). Furthermore, Tascan et al. (2010) found out that skullcap (*Scutellaria spp.*) shoots cultured in liquid media produced the least multiplication ratio and were greatly hyperhydric compared to shoots on agar media. Furthermore, Sreedhar et al. (2009) found that chlorophyll a and b was lost significantly from hyperhydric shoots of vanilla cultured in liquid media in contrast to the normal vanilla shoots cultured in solid (gelled) media. The stomata of hyperhydric vanilla also deprived of closure mechanism and the moisture content were higher compared to normal vanilla shoots. Next, hyperhydric shoots of ornamental cherry tree (*Prunus avium* L.) cultured on gelrite showed reduction of chlorophyll content compared to the normal shoots in agar media (Franck et al. 1998).

Alteration in the concentration of gelling substances is having an impact on the plant reaction. As reported by Kemat et al. (2021), increasing the concentration of gelrite in the media resulted a reduction of HH symptoms in the Arabidopsis seedlings. However, culturing Arabidopsis seedlings in low concentration of agar, gelrite and in liquid media exhibited HH symptoms and increased accumulation of water in the apoplast (Kemat et al. 2023). Furthermore, it was found that as agar concentrations in the media increased, the HH occurrence of *Salvia santolinifolia* Bioss decreased (Jan et al. 2021). Similarly, as agar concentrations added to the media decreased and the incidence of HH rose and lead to the decrement of chlorophyll content of hyperhydric shoots of *in vitro* carnations and French marigold (Modi et al. 2009; Saher et al. 2004). The authors claimed that lack of chlorophyll content in hyperhydric shoots of carnations and French marigold. Next, Yadav et al. (2003) indicated that when the concentration of agar increased, no HH symptoms found in carnation shoots. Ghashghaie et al. (1991) discovered that using low amounts of solidifying agent contributes to an immediate dissolving of gel phase and leads in a totally fluid media during *in vitro* growth.

Besides, Torres and Dangl (2005) documented that the stomatal of leaves grown in high relative humidity do not respond to decreasing water potentials or to darkness. In correspondence with this, it was noticed that the concentration of gelling agents was directly proportional to the water potential of the media. The presence of insufficient gelling agents or an excessive water supply implies that the impact of gelling concentration on plant response could due to alterations in some other gel properties which influence water supply or plant water relations. Other than water potential, capillary action was also found to be the mechanism responsible for pulling water up from the media and distributing it throughout the explant. Capillary action is a crucial factor in the movement of water and the loss of water through transpiration. Rojas-Martinez et al. (2010) reported that capillary forces may be the fundamental mechanism responsible for waterlogging in the apoplast. Therefore, the usage of agar and high concentrations of solidifying agent can decrease capillary actions as well as reduce apoplast flooding, resulting in diminished HH symptoms.

Plant growth regulators (PGRs)

In addition to the environmental prerequisite like light, water, minerals and oxygen, plants also rely on specific chemical substances to transmit, regulate, and facilitate plant growth. These substances are commonly referred as PGRs. PGRs are applied exogenously in the media of *in vitro* culture to induce the desired effect in plant tissues as explants are too small and could not make up the desired natural PGRs required for the growth and development process (Gaba 2004). Soni et al. (2022) described PGRs as chemical compounds that can be produced synthetically or extracted from plant tissue to regulate the growth of cultivated plants and *in vitro* plant cells.

Exogenously applied PGRs enhanced the growth and proliferation rate of plants in tissue culture. Cytokinin (CK) are a significant group of plant growth regulators (PGRs) that have a vital function in the regeneration and proliferation of shoots (Krikorian 1995). Therefore, CK are frequently modified in micropropagation techniques to increase shoot output (Aremu et al. 2014). The induction of hyperhydricity by exogenous CK is often dependent on its concentration (Ivanova and Van Staden 2008). The presence of CK has a significant impact on *in vitro* and HH of horticultural crops, as evidenced by the study conducted by Kadota and Niimi (2003) who found that Thidiazuron (TDZ) and 1-(2-Chloro-4-pyridyl)-3-phenylurea (CPPU), a phenylurea-type resulted in a greater number of hyperhydric shoots in Asian pear (*Pyrus pyrifolia*) as compared to adenine derivatives such as 6-benzyladenine (BA) and Kinetin. Furthermore, media with TDZ in combination with Gibberellic acid (GA_3) also induced HH and stimulated callus formation at high concentrations in culture of Tea Clone Iran compared to 6 Benzylaminopurine (BAP) in combination with GA_3 (Gonbad et al. 2014). These findings are consistent with the study carried out by Kemat et al. (2023) which revealed that TDZ exhibited higher levels of HH and water content in comparison to BAP, Zeatin (Z), and Meta-topolin (MT). Furthermore, the hyperhydric leaves treated with TDZ displayed signs of stress by producing anthocyanins and eventually experiencing leaf necrosis. This indicates that TDZ has a greater capacity to stimulate HH and having a more detrimental effect compared to adenine-type.

Additionally, an experiment conducted by Yadav et al. (2003) resulted that the rate of HH increased when increases the range of combination Kinetin and 1-Naphthaleneacetic acid (NAA) on carnation. Chakrabarty et al. (2006) claimed that apple shoots had visible signs of HH when cultured in media supplemented with BA and Indole-3-butyric acid (IBA) in the bioreactor. The hyperhydric leaves of apple exhibited an uneven epidermal layer characterized by deformed stomata. Similarly, high concentration of BA increased the rate of HH on strawberries whereas NAA reduced HH but callus induction and leaf deformity increased. Furthermore, Gantait and Mahanta (2022) discovered that higher levels of kinetin and BAP produced higher percentage of HH on culture of gerbera (*Gerbera jamesonii* Bolus). Next, the addition of BA in media induced HH syndrome in *Thymus daenensis* shoot cultures where the shoots had high water content, slower growth and less differentiation. The HH symptoms were reversed when BA was removed from the culture media and treated with salicylic acid (SA) (Hassannejad et al. 2012). When exogenous SA is administered in small amounts, it can activate the metabolic defense mechanisms and strengthen the tissue’s resistance to several abiotic stressors (Hayat et al. 2010).

In addition, the chlorophyll content of hyperhydric gerbera and apple cultures also were lesser compared to the controls cultured in media with BAP, kinetin and liquid media with BA and IBA respectively (Gantait and Mahanta 2022;

Chakrabarty et al. 2006). Similarly, chlorophyll a, b and total content in hyperhydric strawberry culture induced by addition of BA in media were notably reduced compared to normal.

Besides, the addition of high CK in media resulted the changes in guard cell (Louro et al 1999) stomata size and density. It is documented by Gantait and Mahanta (2022) and Kemat et al (2023) who found that the stomata size of hyperhydric gerbera were reduced and slightly deformed in shape as compared to the normal gerbera and the reduction of stomatal pore and decreased of stomatal density in hyperhydric Arabidopsis seedlings. Hyperhydric strawberries also have a plasmolysed appearance and reduced deposition of epicuticular wax compared to normal due to the presence of hyperhydric guard cells, which are distorted and hypertrophied. These irregular guard cells have a more circular shape as they are distorted due to the elongation and frequent disruption of the cell wall surrounding the stomatal pore. Lastly, hyperhydric plantlets of China pink exhibited reduced stomatal aperture and a lower stomatal density on their leaves in media without $AgNO_3$ compared to plantlets that were treated in media with $AgNO_3$. These findings suggest that $AgNO_3$ could enhance both the size and number of stomata in hyperhydric plantlets' leaves. The reduced size of the stomatal opening and decreased number of stomata in hyperhydric plantlets may impede the process of transpiration and the evaporation of water inside the plant tissues, resulting in the buildup of water in the tissues (Gao et al. 2017).

Exogenous additives

Supplication of chemical additives has been proven to alleviate HH in tissue culture of various plants. Through meticulous selection and integration of these supplementary elements into the growth media, the quality of plant tissues can be enhaced and overall efficacy of micropropagation can be boosted. Ziv (2008) conducted a study on the effect of silicon (Si) on HH in culture of Star of Bethelehem (*Ornithogalum dubium*) and found out that the addition of Si upgraded the chlorophyll content in the tested leaves and thus, reduced HH problem. Correspondently, Sivanesan et al. (2011) recorded that incorporation of Si from potassium silicate (K_2SiO_3) to Murashige and Skoog (1962) (MS) media reduced the HH in the shoots culture of *Cotoneaster wilsonii* Nakai in contrast with control. This is due to Si capability to develop apoplastic barriers and lignification of xylem elements in plants (Vaculik et al. 2012). Furthermore, Agarie et al. (1998) said that polymerization of Si can support the integrity of the cell membrane and water potential in the epidermal cells of plants and thus, prevent electrolyte leakage. The amount of chlorophyll content in hyperhydric shoots of French marigold (*Tagetes patula* L.) was found to be at lower level than the normal shoots that were supplied with high concentration of agar and NH_4NO_3 in the media (Modi et al. 2009). Next, in a study conducted by Ivanova and Van Staden (2008), it was found that increasing the concentration of ammonium ions in culture media will cause a significant reduction in the incidence of HH of *Aloe polyphylla* shoots. This is due to the detoxification nature of ammonium (NH_4^+) when in abundance that reroute carbohydrates source from metabolic pathway to the pathway of lignin and cellulose production and thus, reduced HH symptoms (Givan 1979). However, too much elevation of ammonium nitrate (NH_4NO_3) concentration that twice of the standard from MS level can induce HH of French Marigold's shoots (Modi et al. 2009). Next, Purohit and Agarwal (2017) tested the effects of NH_4NO_3 at different levels on culture of carnations and it was found that no HH occur when NH_4NO_3 was added in MS media. The addition of nitrate in the media influence in the secondary cell wall composition and structure of plants.

Moreover, a study by Machado et al. (2014) corroborates the finding that exogenous additives of Ca^{2+} plays a role in overcoming HH in English lavender (*Lavandula angustifolia* Mill) where when calcium chloride dihydrate ($CaCl_2.2H_2O$) was applied in the media culture, the occurrence of HH decreased. Ca^{2+} acts as a major part in the cell wall and membranes, as an intracellular messenger in the cytosol and even as a counter-cation for inorganic and organic anions in the vacuole (Marschner 1995). Thus, HH rate in plants culture can be influenced with supplication of Ca^{2+}. Jan et al. (2021), found out that media culture of *Salvia santolinifolia* Bioss shoots that has been added with nitrates from NH_4NO_3, potassium nitrate (KNO_3) and Ca^{2+} from $CaCl_2.2H_2O$ were the best media to reduce HH. HH is linked to the poor cell wall's mechanical structure and according to Hepler and Winship (2010), Ca^{2+} helps in cross-linking negative charges, especially on the carboxylic residues of pectins that resulting in significant structural rigidity to the plant's cell wall, thus can reduce HH problems. Wu et al (2011) had suggested some solutions to reverse the HH symptoms of 'Zhong Sheng Fen' microshoots and allowing the shoots to rejuvenate normally which by removal of NH_4NO_3 from media, addition of activated charcoal (AC) and doubling the concentration of

Ca^{2+} in the media culture.

In addition, Gao et al. (2017) had a subcultured of hyperhydric China pink carnation on media with $AgNO_3$ and found that most hyperhydric plantlets recovered from the state of HH. Furthermore, plantlets cultivated in $AgNO_3$-supplemented media showed noticeable increment of stomatal aperture and density as well as the rate of water loss compared to control. It shows that media supplemented with $AgNO_3$ could give positive effects by alleviating the HH symptoms, as evidenced by their ability to exhibit less HH symptoms and accelerate water loss in China pink carnation. Yadav et al (2003) found that incidence of HH reduced when iron and/or magnesium were added with 0.7% agar on three commercial varieties of carnation namely White sim, Exquisite and Scania. However, iron and/or magnesium concentrations over 0.2 and 3.0 mM might not be optimal for carnation cultivar micropropagation. Therefore, there are significant impact of magnesium and iron and its concentration on HH of carnation culture. The physiological changes on HH development of horticulture crops affected by media components are summarized in Table 1.

Table 1 Effects of media components on physiological changes and HH development in horticultural crops

Horticultural crop	Media components	Physiological changes	References
Arabidopsis (*Arabidopsis thaliana Col-0* and *Ler*)	• Agar and gelrite • Liquid culture media • PGRs: CKs (TDZ, BAP, Z and MT)	- Seedlings showed glassiness, were wrinkled, and had long petioles and larger leaves. Signs of anthocyanin formation were observed on seedlings grown on agar, gelrite, and liquid media with high CK concentrations. - Seedlings grown with TDZ displayed signs of stress by producing anthocyanins and eventually experienced leaf necrosis. - Seedlings grown on agar, gelrite, and liquid media with high CK concentrations had high water accumulation, less air in the apoplast, more water retention in leaves, and less transpiration. - Stomata closure and decreased stomatal density.	Kemat et al. (2023) Kemat et al. (2021)
	• Exogenous additive (*p*-coumaric acid)	- HH occurrence was reduced by inhibiting root growth.	
Gerbera (*Gerbera jamesonii* Bolus)	• PGRs: different CK concentrations (BAP and kinetin)	- High CK concentrations caused HH that reduced the multiplication rate and resulted in deformed leaves and stems. - Hyperhydric leaves appeared glassy, whereas shoots had pale or a yellowish-green color with inward folding of the leaf blade towards the midrib. - Hyperhydric roots were thinner, shorter, and fewer in number. - Less chlorophyll levels, higher relative water content, and reduced stomata size and frequency with deformed guard cells in hyperhydric leaves.	Gantait and Mahanta (2022)
Salvia santolinifolia Boiss	• Different agar concentrations as gelling agent	- Low agar concentrations resulted in wax accumulation on leaves and tissues with a thicker and more translucent appearance.	Jan et al. (2021)
	• PGRs: CKs (BA and 2iP).	- PGRs formed a callus, and shoots had short internodes and abnormal leaves with thick, curled, wrinkled, and fleshy symptoms.	
	• Exogenous additives: NH_4NO_3, KNO_3 and $CaCl_2.2H_2O$.	- HH symptoms were reduced by 23.6% when media was supplemented with NH_4NO_3, KNO_3, and $CaCl_2.2H_2O$. The maximum number of shoots with normal morphology were produced.	
Tea clone Iran 100 (*Camellia sinensis* (L.) O. Kuntze)	• PGR: CKs (BAP and TDZ) and GA_3	- HH symptoms and callus formation in shoots were observed in plants cultured in BAP and GA_3 compared with those cultured in media containing TDZ and GA_3.	Gonbad et al. (2014)

Table 1 Continued

Horticultural crop	Media components	Physiological changes	References
Carnation (*Dianthus caryophyllus* L.)	• Different agar concentrations	- Low agar concentrations induced HH in Exquisite and Scania varieties.	
	• PGRs: auxin (NAA) and CK (kinetin).	- The HH rate in White Sim, Exquisite, and Scania varieties increased as NAA and kinetin media concentrations increased.	Yadav et al. (2003)
	• Exogenous additives: $FeSO_47H_2O$ and NH_4NO_3.	- HH reduced when NH_4NO_3 was added at one-fourth of the overall level. - High addition of NH_4NO_3 resulted in HH in leaves.	Saher et al. (2004) Purohit and Agarwal (2017)
	• Exogenous additives: iron and magnesium	- Iron, magnesium, and 0.7% agar were added to three commercial carnation varieties (White sim, Exquisite and Scania), and the incidence of HH decreased. - However, iron and/or magnesium concentrations over 0.2 and 3.0 mM may not be optimal for carnation cultivar micropropagation.	
China pink (*Dianthus chinensis* L.)	• Exogenous additive: $AgNO^3$	- HH symptoms were reduced and plantlets in $AgNO^3$-supplemented media showed noticeable increase of stomatal aperture, stomata density, and water loss.	Gao et al. (2017)
Strawberry (*Fragaria × ananassa* Duch.)	• PGR: CK (BAP)	- HH rate increased as concentration of media BAP increased. - Antioxidant activity increased, and stomata were malformed as HH symptoms progressed.	Barbosa et al. (2013)
Statice (*Limonium sinuatum* (L.) Mill.)	• Gelrite	- Cultivating shoots in media containing 0.2% gelrite stimulated HH. - The water volume in the leaf apoplast of hyperhydric shoots increased by more than two-fold, and apoplastic air volume in hyperhydric shoots markedly decreased compared with that in non-hyperhydric shoots. - Dysfunctional or aberrant stomata.	Van den Dries et al. (2013)
Watermelon (*Citrullus lanatus* (cv. Giza1))	• Agar and gelrite	- HH was more severe in media with gelrite as the gelling agent than in agar.	Badr-Elden et al. (2012)
	• PGRs: CK (different BAP concentrations)	- Increasing BAP concentration led to higher shoot regeneration but more pronounced HH was observed. - Hyperhydric shoots were thicker than normal shoots.	
Thymus daenensis Celak	• PGRs: CK (BAP) and SA	- BAP induced HH symptoms where shoots had high water content, slower growth, and less differentiation. - SA reversed HH symptoms.	Hassannejad et al. (2012)
Zhong Sheng Fen (*Paeonia lactiflora* Pall.)	• PGRs: CK (BAP) and auxin (IAA, IBA, and NAA)	- BAP induced HH where shoot stems were more transparent with curled leaves. - NAA reduced HH; however, callus induction and leaf deformity increased.	Wu et al. (2011)
	• Exogenous additives: NH_4NO_3, AC, and Ca^{2+}	- Eliminating NH_4NO_3, adding AC, and doubling Ca^{2+} in culture media reversed HH symptoms.	
Cotoneaster wilsonii Nakai	• Exogenous additive: Si	- Hyperhydric shoots had malformed and wrinkled leaves with a glassy and translucent appearance. - Si in media helps to reduce HH compared to control.	Sivanesan et al. (2011)
Spiral Aloe (*Aloe polyphylla Schönland ex Pillans*)	• Agar and gelrite	- Hyperhydric shoots had thicker, translucent, and water-logged leaves compared with those of normal shoots. - Low HH incidence and high shoot regeneration were observed in media with agar compared with media with gelrite.	Ivanova and Van Staden (2011)
	• PGRs: CKs (BAP, zeatin, and TDZ)	- BAP and zeatin induced HH.	Ivanova and Van Staden (2008)
	• Exogenous additive: ammonium ions	- The incidence of hyperhydric shoots was reduced when ammonium was added to media.	

Table 1 Continued

Horticultural crop	Media components	Physiological changes	References
Skullcaps (*Scutellaria*)	• Liquid culture media	- Plants cultured in liquid media were greatly hyperhydric compared with plants cultured in agar gel and liquid with polyester fiber-supported paper media. - The multiplication rate decreased as the HH rate increased. - Non-hyperhydric plants produced the greatest percentage of dry weight, and HH was linked to water uptake.	Tascan et al. (2010)
Star of Bethelehem (*Ornithogalum dubium* Houtt.)	• Exogenous additive: Si	- Si helped to increase the chlorophyll content and consequently alleviated HH symptoms.	Ziv (2008)
French Marigold (*Tagetes patula* L.)	• Different agar concentrations • Exogenous additive: NH_4NO_3	- HH was caused by stress and resulted in low chlorophyll concentrations. - HH decreased as agar concentrations increased (0.9-1.5%). - NH_4NO_3 addition alleviated HH symptoms, but too much NH_4NO_3 (twice the MS level) induces HH. - An NH_4NO_3 concentration of 10.3 mM in media culture is most effective against HH.	Modi et al. (2009)
Vanilla (*Vanilla planifolia* Andrews)	• Liquid culture media	- Loss of chlorophyll content in hyperhydric leaves. - The moisture content of hyperhydric vanilla shoots is higher than that in normal vanilla shoots. - Closure mechanism of stomata was absent in hyperhydric leaves.	Sreedhar et al. (2009)
Apple (*Malus* × *domestica* Borkh.)	• Liquid culture media • PGRs: CK (BAP) and auxin (IBA)	- Apple shoots (28%) had visible signs of HH. Hyperhydric leaves exhibited an abnormal appearance with an uneven epidermal layer and stomata malformation where they were raised above the surface of the leaf and fully visible. - The leaves with HH contained more water and less chlorophyll than that in healthy leaves.	Chakrabarty et al. (2006)
Ornamental cherry (*Prunus avium* L.)	• Agar and gelrite	- Hyperhydric shoots cultured on gelrite showed lower chlorophyll content than that in normal shoots cultured on agar media.	Franck et al. (1998)

HH, hyperhydricity; PGR, plant growth regulator; CK, cytokinin; TDZ, thidiazuron; BAP, benzylaminopurine; Z, zeatin; MT, meta-topolin; 2iP, 2-isopentenyladenine; GA_3, gibberellin A3; NAA, naphthaleneacetic acid; SA, salicylic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid.

Anatomical and Biochemical Effect of Media Components

Leaves are vital parts of plants that acts as spaces for various fundamental processes in plants such as photosynthesis and transpiration. The anatomical structure of leaves can diverse significantly in response to stress conditions similar to the conditions of *in vitro* culture. One such diversion noted in hyperhydric leaves is the abnormal accumulation of water. Furthermore, the anatomical observation of normal leaves plants would show well-shaped, sized and compacted cells with established arrangement in the cortex area. However, the cells arrangement in the cortex cells was disoriented and deformed in hyperhydric plantlets of gerbera when concentrations of BAP and kinetin applied in media were increased (Gantait and Mahanta 2022). Kemat et al. (2023) also found out that normal leaves of Arabidopsis that are grown in media with no CK added and agar as solidifying agent had a distinct dorsiventral homogenous mesophyll which a single layer of epidermis and palisade present and leaf epidermis has thin cuticle. The collateral vascular bundles were also present and observed. However, hyperhydric Arabidopsis that were induced through media with gelrite and different types of CK (BAP, TDZ, Zeatin, MT and CPPU) exhibited disorganized mesophyll which where the cells are globular and a distinct of palisade parenchyma was absent. Besides, there was presence of substantial intercellular gaps and the lack organization of vascular bundles can be observed.

Besides, Franck et al. (1998) discovered that the stems of hyperhydric shoots ornamental cherry tree (*Prunus avium* L.) lacked of sclerenchymatic areas, had fewer developed and lignified xylem tissue and showed hypertrophy of the cortical parenchyma when plants were cultured with gelrite. Moreover, Sreedhar et al. (2009) found out that normal vanilla shoots that were cultured in solid (gelled) media showed a xerophytic morphology with succulent waxy leaves but hyperhydric vanilla shoots that were cultured in liquid media showed clear disintegration of the endodermal cells. Also, the leaves of hyperhydric vanilla cultured in liquid media displayed lack of vascular bundles, reduce of compactness of the palisade parenchyma with abnormal enlargement and more intercellular space compared to normal.

Next, Chakrabarty et al. (2006) induced HH buy culturing apple in liquid media supplemented with BA and IBA and observed a thin and discontinuous cuticle with less epicuticular wax in hyperhydric leaves as compared to the healthy ones. Healthy leaves possess a distinct palisade layer consisting of a single layer of cells, as well as a spongy mesophyll. In contrast, hyperhydric leaves lack a palisade layer and only contain a spongy mesophyll with significant disorganized intercellular gaps. The results were similar as in the comparative studies of hyperhydric and normal strawberry cultivated in vitro by Barbosa et al. (2013). It was revealed that normal strawberry exhibited a distinct dorsiventral mesophyll structure, consisting of a single layer of palisade parenchyma and two to three cell layers of spongy parenchyma with minimal intercellular gaps. With an increase in BA concentration in the media culture, HH was induced and hyperhydric strawberry showed no distinction between spongy and palisade parenchyma, exposing enlarged and round cells along with a wider leaf lamina. Hence, HH on plants can dissociate the normal anatomical composition of plants such as disoriented and deformed of cell arrangements in their cortex area, lack of vascular bundles, reduced compactness of the palisade parenchyma and messy vascular bundle organization were exhibited.

Understanding the biochemical changes caused by HH provides deeper insights into how HH impacted the function and health of plants culture. According to Polivanova and Bedarev (2022), BA is beneficial as it can escalate peroxidases activity and consequently elevate lignification in plants. Contrary, Mamedes-Rodrigues et al. (2019) mentioned that BA induced HH but did not affect lignification pathway when treated with gas exchange in *Brachypodium distachyon.* Lignin is one of the components in the cell wall's apoplast and the biosynthesis of lignin consists of a long process but initially started from the general phenylpropanoid pathway where deamination of amino acid phenylalanine happened to form cinnamic acid, followed by p-coumaric acid and p-coumaroyl-CoA (Labeeuw et al. 2015). The lignin content in plant cell walls plays a significant role in preventing HH symptoms. Saher et al. (2004) recorded higher degree of lignification when carnations were cultured in higher concentration of agar and it contribute to positive effect on preventing HH symptoms in carnation shoots. This is supported by Kemat et al. (2021) where found the total lignin content of Arabidopsis culture was increased when treated with *p*-coumaric acid that consequently decreased the symptoms of HH. Besides, Kumsa (2016) said that the presence of liquid and low agar media can enhance the development of HH by stimulating cellulose production and interfering with the regular process of cellulose biosynthesis in grapes.

Other than lignin, pectin is a polysaccharide and one of the major components in plant cell walls. It has multiple uses in plants including the stimulation of lignification biosynthesis which is important in combating HH (Xiao et al. 2017). Saher et al. (2005) claimed that when decreased the concentration of agar less production of total pectin content compared to controls in culture of carnation. The pectin production is regulated by the activity of pectin methyl esterases (PME). According to Goldberg et al (1996), PMEs can follow the pectin chain in a linear or random manner. The random manner can cause de-esterification and loosens of cell while the linear action encourages Ca^{2+} cross-linkages, releasing free carboxyl groups for pectate gel formation. Therefore, it was found that PME activity of hyperhydric carnation was higher compared to controls as to regulate some of the structural changes related to HH.

Besides, Purohit and Agarwal (2017) found that reducing NH_4NO_3 at one-fourth of MS level can reduce the occurrence of HH of carnation culture where the antioxidant enzyme such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) activity decreased following decreasing number of hyperhydric shoots. These CAT, SOD and POD enzymes protect cells from oxidative damage by scavenging reactive oxygen species (ROS) and converting reactive superoxides into oxygen and water (Saffar et al. 2009). Gao et al. (2017) found that $AgNO_3$ significantly reduced ROS levels and superoxide production rates in hyperhydric plantlets grown in $AgNO_3$-supplemented media. This reduced ROS levels improved antioxidant capacity. The study also examined the impact of $AgNO_3$ on genes related to ethylene production and signal transduction, finding lower expression levels in $AgNO_3$-supplemented plants compared to those without $AgNO_3$ supplementation. This suggests that $AgNO_3$ can improve antioxidant capacity and ethylene production, potentially reducing HH symptoms. Similar results can also be observed in culture of snapdragon (Lee et al. 2023).

Furthermore, Hassannejad et al. (2012) observed that polyamines (PAs) levels were different between hyperhydric and normal culture of *Thymus daenensis.* PAs level of hyperhydric shoots cultured in media with BA was lesser compared to normal shoots cultured in media with SA. PAs are connected with cell wall components (lignin and hemicellulose) and the membrane proteins of chloroplasts.

The decreased concentration of PAs in hyperhydric shoots may be connected to the loss of cell wall and chloroplast integrity, which is a defining feature of HH. The anatomical and biochemical changes on HH development of horticulture crops affected by media components are listed in Table 2.

Molecular Effects of Media Components

Comprehending the molecular impacts of HH on plants is essential for optimising the appropriate components of media components and improving plant regeneration strategies. Bakir et al. (2016) found that the transcriptome analysis

Table 2 The effects of media components on anatomical and biochemical changes and HH development in horticultural crops

Horticultural crop	Media components	Anatomical and biochemical changes	Source
Arabidopsis (*Arabidopsis thaliana Col-0* and *Ler*)	• PGRs: CKs (BAP, TDZ, zeatin, MT and CPPU) • Exogenous additive (*p*-coumaric acid)	- Hyperhydric seedlings showed disorganized mesophyll (globular cells and absence of distinct palisade parenchyma.) - Presence of substantial intercellular gaps and lack of vascular bundle organization in hyperhydric shoots. - Increased total lignin in *Arabidopsis* seedlings.	Kemat et al. (2021) Kemat et al. (2023)
Gerbera (*Gerbera jamesonii* Bolus)	• PGRs: different concentrations of CKs (BAP and kinetin).	- Increased PGR concentrations resulted in disoriented and deformed cells in cortex area of hyperhydric plants.	Gantait and Mahanta (2022)
Carnation (*Dianthus caryophyllus* L.)	• Exogenous additive (NH_4NO_3) • Different agar concentrations	- Antioxidant enzyme (CAT, SOD and POD) activity increased following an increase in the number of hyperhydric shoots. - Lignin content in hyperhydric shoots was lower than that in normal shoots. - Pectin content reduced as HH symptoms became more severe. - PME activity in hyperhydric leaves was higher than that in controls.	Purohit and Agarwal (2017) Saher et al. (2005) Saher et al. (2004)
China pink (*Dianthus chinensis* L.)	• Exogenous additive ($AgNO_3$)	- Supplementing $AgNO_3$ in media decreased ROS levels in plants and improved antioxidant capacity, thereby alleviating HH. - $AgNO_3$ reduced ethylene production, thereby alleviating HH.	Gao et al. (2017)
Strawberry (*Fragaria x ananassa Duch. 'Dover'* and *'Burkley'*)	• PGR: CK (BAP)	- Hyperhydric strawberry showed no distinction between spongy and palisade parenchyma, cells were enlarged and round, and the leaf lamina was wide. Normal crops a had a normal single layer of palisade parenchyma and two to three cell layers of spongy parenchyma with minimal intercellular gaps. - Hyperhydric strawberry had a plasmolyzed appearance and reduced deposition of epicuticular wax. - Strawberries with HH showed irregular guard cells that were distorted and hypertrophied. - Activity of the antioxidant enzymes POD and CAT in hyperhydric strawberries increased in direct proportion with the culture medium BAP concentration.	Barbosa et al. (2013)
Thymus daenensis Celak	• PGRs: CK (BAP) and SA	- PA levels in hyperhydric shoots cultured in media with BAP were lower than that in normal shoots cultured in media with SA.	Hassannejad et al. (2012)
Vanilla (*Vanilla planifolia* Andrews)	• Liquid culture media	- Hyperhydric leaves lacked vascular bundles and compactness of the palisade parenchyma with abnormal enlargement and large intercellular spaces.	Sreedhar et al. (2009)
Apple (*Malus × domestica* Borkh.)	• Liquid culture media • PGRs: CKs (BA and IBA)	- Thin and discontinuous cuticle with less epicuticular wax in hyperhydric leaves than that in healthy leaves. - Hyperhydric leaves lacked a palisade layer and only contained a spongy mesophyll with significant disorganized intercellular gaps. - Antioxidant enzyme activities were found at higher levels in hyperhydric apple leaves than in normal apple leaves.	Chakrabarty et al. (2006)
Ornamental cherry tree (*Prunus avium* L.)	• Gelrite as gelling agent	- Lacked sclerenchymatic areas and had fewer developed and lignified xylem tissues in hyperhydric leaves. - Hyperhydric cherry exhibited hypertrophy of the cortical parenchyma.	Franck et al. (1998)

HH, hyperhydricity; PGR, plant growth regulator; CK, cytokinin; TDZ, thidiazuron; BAP, benzylaminopurine; Z, zeatin; MT, meta-topolin; CPPU, forchlorfenuron; SA, salicylic acid; IBA, indole-3-butyric acid; POD, peroxidase; CAT, catalase; SOD, superoxide dismutase; PME, pectin methylesterase; ROS, reactive oxygen species; PA, polyamine.

of gene expression changes between hyperhydric peach shoots compared to normal one. HH was developed when the normal shoots were transferred from agar to gelrite media. There were a downregulation of ABC transporters and transcription factors observed. The downregulation of the ABC transporters in the hyperhydric leaves impacted the cuticle development and stress-responsive genes. HH is concluded as a complicated biological process that may be observed at the molecular level through changes in the expression of several transcripts by RNA-Seq in conjunction with rigorous statistical analysis.

Futhermore, Chakrabarty et al. (2006) found that glutathione S-transferase (GST) and dehydroascorbate reductase (DHAR) activities in apple culture decreased in response to HH conditions. The HH was induced by using liquid culture media supplied with BA and IBA. GSTs are crucial for detoxification, facilitating glutathione conjugation to harmful compounds. The decrease in GST activity suggests GST is not engaged in detoxification in hyperhydric apple leaves, while the decrease in DHAR activity suggests the inactivation or breaking of peroxidative enzyme.

Picoli et al. (2008) has conducted a protein extraction and quantification, SDSPAGE electrophoresis and immunoblot analysis on leaves sample of *Eucalyptus grandis*. HH of Eucalpt were observed as plants were cultured in agar media supplied with BAP and NAA. It was found out that the molecular chaperone, binding immunoglobulin protein (BiP) was expressed more intensively in hyperhydric Eucalpt compared to normal one. The result of enhanced BiP expression in hyperhydric Eucalypt is expected as Cascardo et al. (2000) said that, BiP expression was related to abiotic stress including water stress as HH is hugely related to immoderate water availability in plants.

Furthermore, a proteomic analysis between control and hyperhydric carnation was conducted by Muneer et al. (2017) by analysing through SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) in addition of Si. It was found that Si alleviated the symptoms of HH and the protein folds showed the same results as the control. Moreover, most of the protein spots that were detected had a significant relationship to stress response, photosynthesis, and signal transduction using gene ontology and the rest were classified as proteins that involved in either primary or secondary metabolic processes. Through the study, Si turned out helpful in regulating the protein synthesis, photosynthesis and stress response as the application of it showed the alleviation of carnation towards HH. The molecular impacts of media components are summarised in Table 3.

Table 3 The molecular effects of media components on horticultural crops

Horticultural crop	Media components	Result	Source
Carnation (*Dianthus caryophyllus* L.)	• Exogenous additive: Si	- Protein folds in hyperhydric carnations were either upregulated or downregulated compared with those in control carnations. - Si was applied to alleviate HH and the protein folds showed the same results as the control with no HH. - Overall, 900 protein spots were observed on the 2DE maps and 70 protein spots were differentially expressed among them. - Protein spots that were detected had a significant relationship with stress response, photosynthesis, and signal transduction according to gene ontology and the rest were classified as proteins that were involved in either primary or secondary metabolic processes.	Muneer et al. (2017)
Peach (*Prunus persica* var. *nectarina* (Aiton))	• Gelrite as gelling agent	- Transcriptome analysis of gene expression changes between hyperhydric peach shoots and normal shoots was conducted. - Downregulation of ABC transporters and transcription factors were observed in hyperhydric leaves and affected cuticle development and stress-response genes. - RNA-Seq and statistical analysis concluded that HH was observed at the molecular level through changes in the expression of several transcripts.	Bakir et al. (2016)
Eucalyptus (*Eucalyptus grandis* (Hill) Maiden)	• PGRs: CK (BAP) and auxin (NAA)	- Molecular chaperone, binding immunoglobulin protein (BiP) was expressed more intensively in hyperhydric Eucalpytus leaves than in normal Eucalpytus leaves. - Increased BiP expression in hyperhydric Eucalyptus was expected because BiP expression was related to abiotic stress.	Picoli et al. (2008)
Apple (*Malus* × *domestica* Borkh.)	• Liquid culture media • PGRs: CK (BAP and IBA)	- GST and DHAR activities decreased in response to HH.	Chakrabarty et al. (2006)

HH, hyperhydricity; PGR, plant growth regulator; BAP, benzylaminopurine; NAA, naphthaleneacetic acid; SA, salicylic acid; IBA, indole-3-butyric acid; ABC, adenosine triphosphate-binding cassette; RNA-Seq, RNA sequencing; GST, glutathione S-transferase; DHAR, dehydroascorbate reductase.

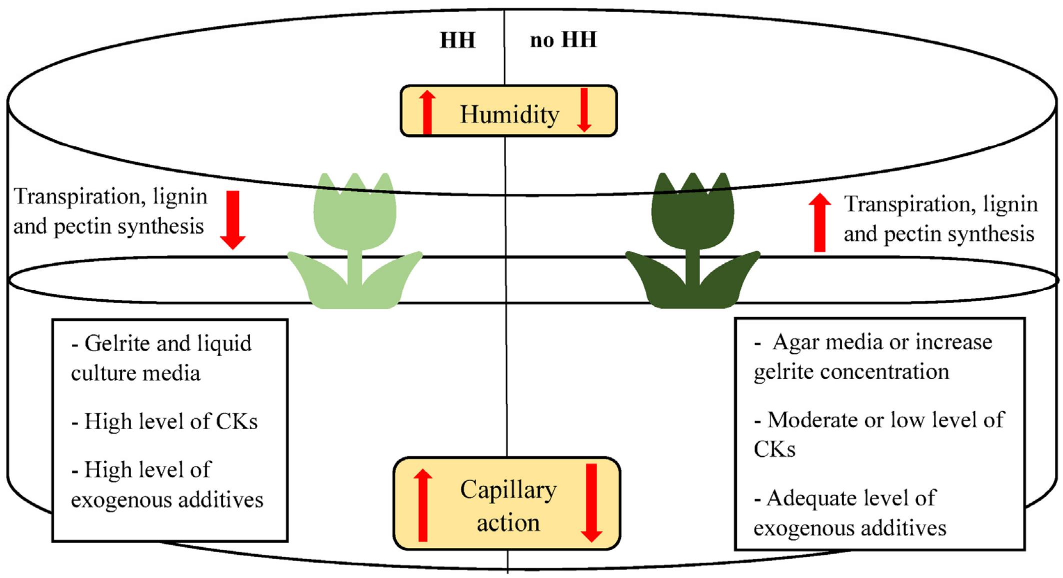

Fig. 1 Schematic summary of the effects of media components on hyperhydricity (HH) in horticultural crops. (left) Interaction of the explants with the media components (gelrite, liquid media, and high cytokinin [CK] and additive concentrations) and movement driven by capillary action increases water potential, thereby resulting in HH. These conditions result in physiological, anatomical, biochemical, and molecular changes, such as decreased transpiration and synthesis of lignin and pectin, thereby resulting in HH. (right) HH symptoms can be reduced or alleviated by using agar, increasing gelrite concentration (less water potential), applying moderate or low CK levels, and adding adequate concentrations of exogenous additives, such as Si, nitrate, ammonium ions, Ca^{2+}, p-coumaric acid, or $AgNO_3$. This will also increase transpiration and synthesis of lignin and pectin

Conclusion

The current review analysed primary factors of media components in HH development of horticultural crops. The degree of HH developed is determined not only by the concentrations of the hormones, but also by the type of hormones. Like CK, the impact of gelling agents such as agar, gelrite, and liquid media on the amount of HH was also found in numerous horticultural crops. These findings indicated that the concentration of gelling agents was directly proportional to the water potential of the medium. Addressing HH is a crucial aspect of developing a successful tissue culture technique. We also pointed out the effect of exogenously applied compound in alleviating HH of horticultural crops. Addition of compound reduces the symptoms of HH by increase lignin, pectin and protein level. A schematic depiction (Fig. 1) showed the effect of media components (PGRs and gelling agents) on HH and normal plants. Implementing any of the recommended approaches to alleviate HH may not always effective. Different plant species may have specific requirements for the type of growing media and cultural environment. Hence, it is crucial to comprehensively examine and grasp the phenomenon of HH for each individual species.

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