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IRON FORMULATION AFFECTS *IN VITRO* STORAGE OF HOPS: AN IMAGE ANALYSIS

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Summary

In vitro-stored plant germplasm is usually evaluated by visual analysis of the plant based on subjective characters. To reduce the variability in these evaluations, we developed a digital-image evaluation system for *in vitro*-stored plantlets. This study compares the standard visual evaluation system with a digital analysis system to determine if digital analysis can effectively quantify the health of diverse *Humulus* germplasm. Eight cultivars of *Humulus lupulus* L. were stored on standard Murashige and Skoog (MS) medium with iron alone (EDTA chelated) and on MS iron with 100 or 200 mg l^{-1} sequestrene 138 iron (EDDHA chelated). Digital images of the upper two nodes of each plantlet were evaluated for red, green, blue, green/red ratio, and modified normalized difference vegetation index (MNDVI = R − G/R + G). Evaluation of each plantlet for MNDVI values showed consistent significant differences for all treatments only at the upper node. Significant differences for visual and the MNDVI values among the three iron treatments were observed at the upper node of most of the eight hop cultivars. Regression analysis of the upper node MNDVI values vs. whole-plant visual ratings showed positive correlations for most cultivars. Effects of iron treatments on storage duration were also analyzed for both visual and digital systems. There were significant differences among MNDVI values for plantlets stored on medium with standard MS iron alone (EDTA chelated) and with the addition of sequestrene 138 iron. In general, the MNDVI value of the upper node correlated well with visual ratings and could be used to determine the health of *in vitro* stored hops.

Key words: cold storage; germplasm; hops; *Humulus*; image analysis; micropropagation.

Introduction

The U.S. Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository maintains a 700 accession *Humulus* (hops) germplasm collection (Reed et al., 2003). These accessions are grown in field collections, in pots in the screen house, as tissue cultures in cold storage, and as cryopreserved shoot tips in liquid nitrogen. The storage longevity of the *in vitro* collection depends on many important factors (Ashmore, 1997). *In vitro* containers (Reed, 1991, 1992), cold acclimation (Reed, 1993) nitrogen concentration (Moriguchi and Yamaki, 1989), growth regulators (Reed, 2002) and the medium composition can all affect longevity in storage. Reed et al. (2003) found that *Humulus* plantlets on Murashige and Skoog (MS) iron remained viable in cold storage for an average of 14.1 ± 3.5 months. The viability of individual accessions varied during storage from 6 to 26 months, and cultivar storage was similar (14.6 ± 3.4) to that of the wild accessions (12.6 ± 3.2). Variation occurred between storage cycles due to many factors. When compared to data from a 2003 study, the eight genotypes included in both studies remained viable in storage, with ratings >2, from 9 to 21 months. The usual iron used in *Humulus* culture medium is based on the MS formulation (Murashige and Skoog, 1962). Sequestrene iron is used in plant growth media for various purposes (Van der Salm et al., 1994; Castillo et al., 1997; Tsao and Reed, 2002). We recently determined that culture medium with sequestrene iron greatly improved leaf color and growth of many species and cultivars of hops. (Reed, unpublished).

Image analysis is used for many scientific studies and industrial applications. Color analysis is used to study plant cells or biological products in such widely varied plant systems as wheat senescence (Adamsen et al., 1999) and fruit grading to determine picking time for apples (Schrevens and Raeymaeckers, 1992).

The goal of this study was to determine if a newly developed image-analysis procedure developed to detect deterioration for pear shoot cultures (Aynalem et al., 2006) could be applied to cultures of *Humulus* germplasm and if it could be applied to experimental evaluations. Image analysis was compared to visual observations in an experiment designed to determine if the improved vigor of growth-room plantlets on medium with sequestrene iron would be maintained during cold storage and whether the mean storage duration would change with changes in the iron content of the medium.

Materials and Methods

Plant materials. Eight hop (*Humulus lupulus*) cultivars were used in this study: Alpha Aroma, Hallertauer Tradition, Hersbrucker-8, Mt. Hood, Pacific Gem, Spalter Select, USDA 21119 and Vojvodina.

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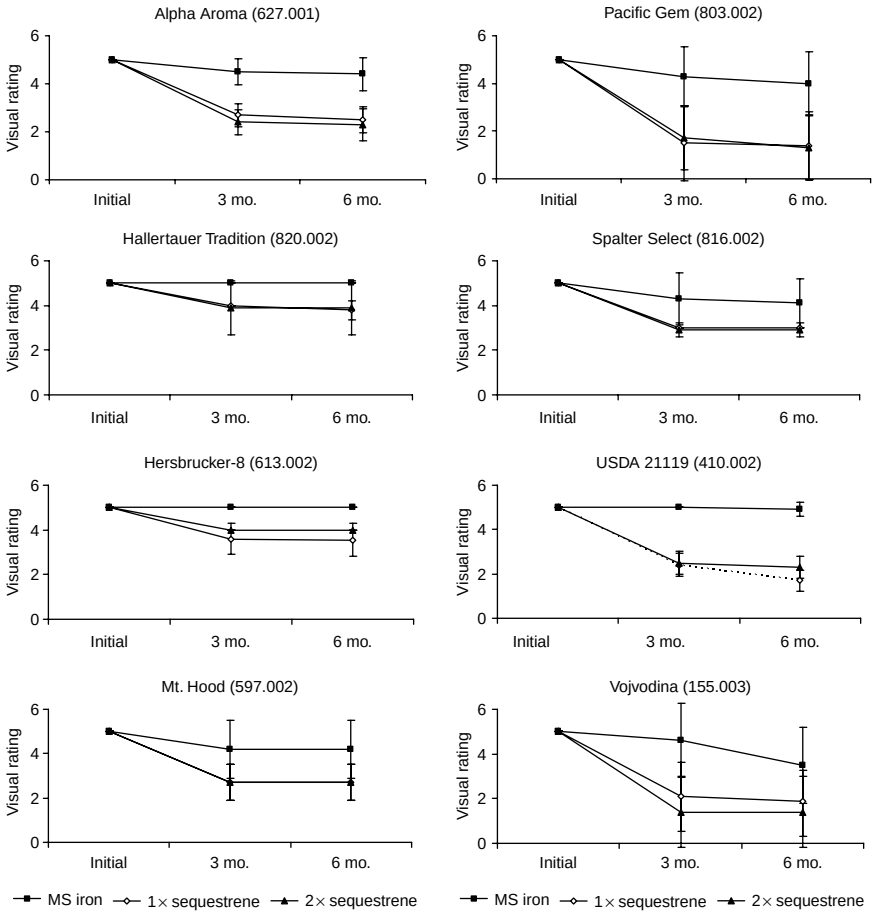

FIG. 1. Storage condition ratings of eight *Humulus* genotypes after 0 (initial), 3, or 6 mo. of 4°C cold storage on NCGR-HUM medium with EDTA chelated iron (MS formulation) alone or MS iron and 100 mg (1 ×) or 200 mg (2 ×) of EDDHA chelated iron (sequestrene 138) added per liter (Aynalem and Reed, 2005). Means ± SD of five replications (plantlets).

Culture conditions. *In vitro* cultures were originally initiated from 0.3–0.5 mm meristems of heat-treated shoots from clonally propagated hop plants (Reed et al., 2003). Plantlets were grown on NCGR-HUM medium [MS (Murashige and Skoog, 1962) salts and vitamins with 2% glucose, 4.4 μM N^6 benzyladenine, at pH 5.0 and solidified with 0.3% agar and 0.125% Gelrite] before use in this study. Shoots were multiplied on 40 ml of medium in Magenta GA-7 vessels at 25°C under a 16-h photoperiod (40 $\mu mol\, m^{-2}\, s^{-1}$).

Cold storage of in vitro *cultures*. Plantlets with two nodes (2–3 cm height) were transferred to five-chambered semi-permeable tissue-culture bags (StarPac, Garner Enterprises, Willis, Texas), 10 ml medium per chamber, 3 wk after the last regular subculture. Standard NCGR-HUM storage medium was tested with three iron sources (described below), without growth regulators, and with 0.35% agar and 0.145% Gelrite. Five plantlets of each accession were prepared for each treatment with each plantlet in an individual section (15 × 150 mm) of a five-section bag.

Cold acclimation. Plants in bags were grown for 1 wk under growth-room conditions, then cold acclimated for 1 wk in a growth chamber with temperature/photoperiod settings of −1°C 16-h dark/22°C 8-h light (10 $\mu mol\, m^{-2}\, s^{-1}$) as the standard treatment (Reed et al., 1998).

Experimental treatments. Each of the eight accessions was put into cold storage on three iron treatments: standard MS iron (EDTA chelated) or MS iron with an additional 100 or 200 $mg\, l^{-1}$ sequestrene iron 138 (EDDHA chelated). Each treatment consisted of two bags with five plantlets each. Plantlets were stored at 4°C with a 12-h photoperiod and very low light (3 $\mu mol\, m^{-2}\, s^{-1}$).

Visual evaluation. Hop plantlets were evaluated at storage and at 3 and 6 mo. Each plantlet was rated on a 0–5 scale. Ratings were: 5, dark green leaves and stems, no etiolation, base green; 4, green leaves and stems, little etiolation; 3, shoot tips and upper leaves green, etiolation present, base green; 2, shoot tip green, leaves and stems mostly brown, base may be brown; 1, plantlet mostly brown, only extreme shoot tip green, much of base dark brown; 0, all of plantlet brown, no visible green on shoot tip. Plantlets are normally repropagated when ratings drop to 2 or lower.

Image analysis. The computer-image vision system employed a digital camera with 4 mega pixels and 35 mm compact/zoom. The camera zoom lens was set to a standard height (30 cm) above the viewing surface. The area viewed by the camera was adjusted to the area of the bags (22.5 × 15 cm). The bags with stored plantlets were placed on the *trans*-illuminator (slide viewer). The camera was set with a shutter speed of 1/500 s and aperture at f/4.0.

Image analysis software was available from web sites and included Graphic workshop (www.geo.oregonstate.edu/esri/), Arc Explorer (www.esri.com/software/arcexplorer/) and ArcMap (www.mindworkshop.com/alchemy/gwspro.html). Data Crunching Center (DCC) (developed in the Department of Horticulture, Oregon State University) and Microsoft Excel were also used. The illumination system consisted of a light stand and a supporting surface for the samples. The illumination was produced by four fixed lamps (Watt-Miser indoor reflector flood, 120 watts each) from the right and left sides. The lights on the same side were 45 cm apart and 98 cm from the lights on the opposite side. The white background with back lighting (20 ME $m^{-2}\, s^{-1}$) provided high contrast with the plantlets inside each cell.

Image acquisition and processing. Color images were represented as 24-byte images with red, blue and green bands. Images were cropped and scaled with Graphic Workshop software. The image of each plant was isolated from the background and stored as a Tag Image Format File (TIFF) with about 1000 x 500 pixels. A pixel represented 0.2 mm. Images were transferred to

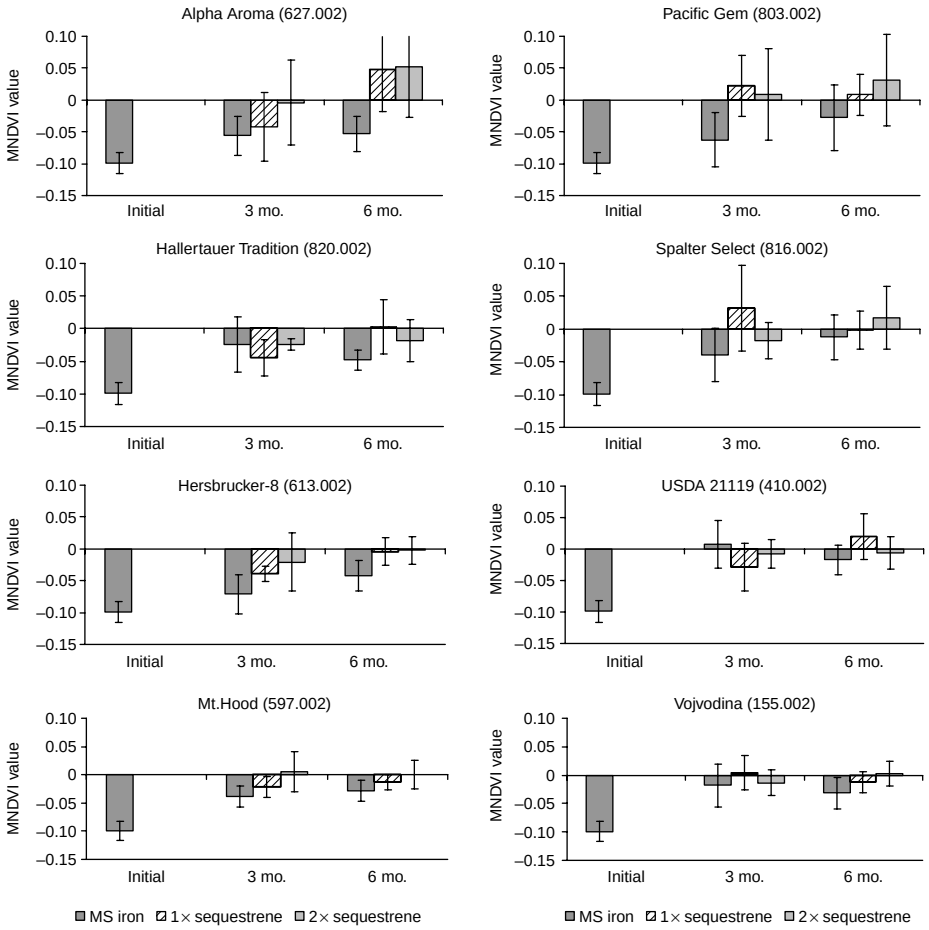

FIG. 2. Mean modified normalized vegetation index (MNDVI) values from digital photos of the upper node of *in vitro* cultures of four hop (*Humulus lupulus*) cultivars stored at 4°C for 0, 3 or 6 mo. on NCGR-HUM medium with EDTA chelated iron (MS formulation) alone or MS iron with 100 or 200 mg of EDDHA chelated iron (sequestrene 138) added per liter compared with the control (initial, 0 mo.). *Bars* represent means ± SD of five replications (plantlets).

ArcMap and a polygon was created around the sampled node consisting of 10–15 pixels for each polygon sampled. Sampling areas were at the same place on each node for each plantlet throughout the experiment (following the node during the growth of the plant, while in storage). Pixels within the sample shape files were retrieved with DCC software and converted to a database. The mean values for blue, green and red in each image were calculated using Microsoft Excel. The RGB values were converted to Hue, Intensity and Saturation (HIS) and modified normalized difference vegetation index, MNDVI (R − G/R + G) for additional analysis. The MNDVI is similar to the NDVI used with near infrared imagery, but modified for use with visible wavelengths.

Statistical analysis. The two top nodes of five plantlets of each cultivar were analyzed using a similar number of pixels in the sampling points. Eight different pixel expressions; red, green, blue and ratios MNDVI (R − G/R + G), green to red ratio (G/R), and hue saturation and intensity were analyzed. The means and standard deviations were computed for all eight pixel value distributions for combined and individual nodes using the pivot table feature in Microsoft Excel. The image evaluation data were compared with visual evaluation ratings using regression analysis.

RESULTS AND DISCUSSION

Visual evaluation. Ratings of hop plantlets stored on MS iron were equal or better than ratings of plantlets with added sequestrene iron at 3 and 6 mo. (Fig. 1). 'Alpha Aroma', 'Pacific Gem', USDA 21119, and 'Hersbrucker-8' on MS iron maintained ratings > 4 at 6 mo. and performed significantly better than plantlets with sequestrene iron. All genotypes maintained mean ratings >3 when stored for 6 mo. on MS iron alone. Half of the eight genotypes stored on sequestrene iron were rated near 2 at 6 mo., indicating a need for repropagation. Given the trend shown in these data, it is likely that plantlets of most accessions stored with added sequestrene iron would be dead at 12 mo., while those on MS iron would remain in storage considerably longer (Fig. 1). In an earlier study, we found that *Humulus* plantlets grown on MS iron remained viable in 4°C cold storage for 14.1 ± 3.5 mo. with a range of 6–26 mo. (Reed et al., 2003). These cultivars stored for 14.6 ± 3.4 mo. and wild accessions for 12.6 ± 3.2 mo. The study in 2003 found 'Hallertauer Tradition' and 'Hersbrucker-8' remained viable in storage for 17 mo. and 'Mt. Hood' for 11 mo. These values are consistent with the high ratings from visual evaluations of these accessions stored on MS iron at 6 mo. (Fig. 1).

Image analysis. Evaluation of the two nodes of each plantlet and the combined mean MNDVI values of the nodes showed consistently significant differences among the treatments only for

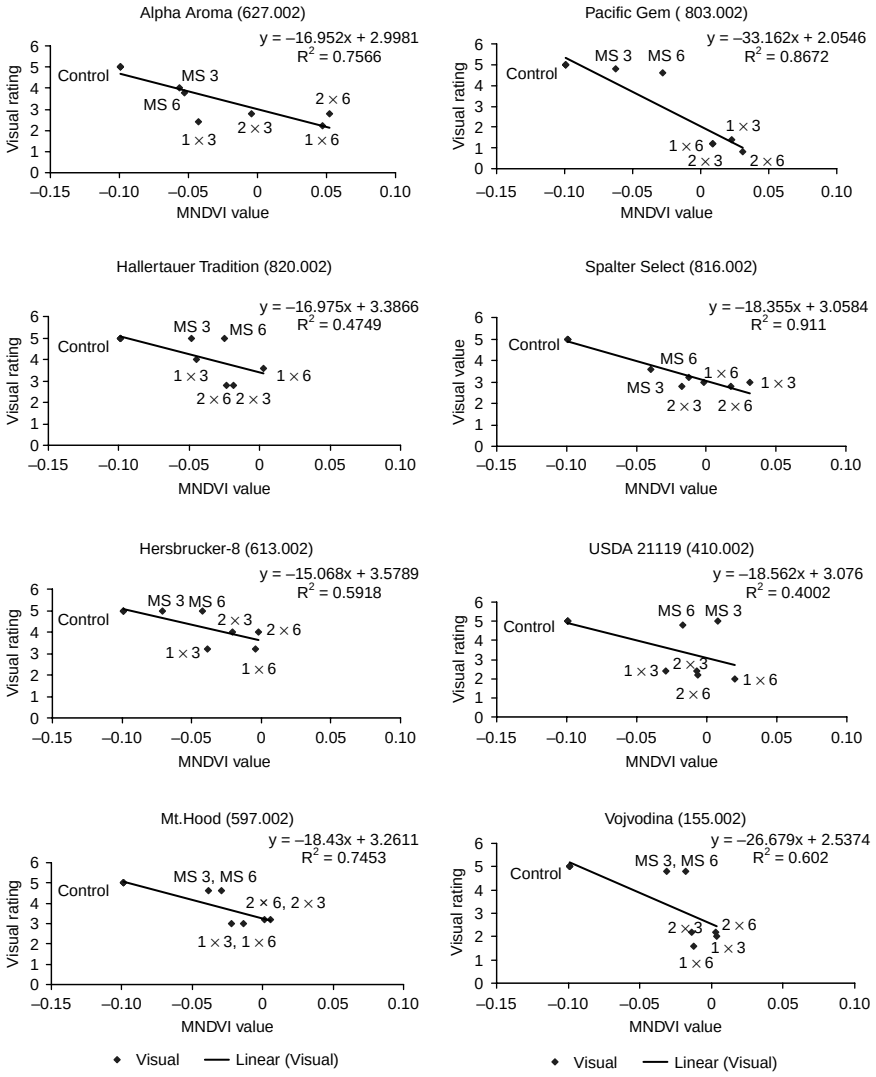

FIG. 3. Regression analysis of the upper node MNDVI values vs. visual ratings for eight hop cultivars on NCGR-HUM medium with EDTA chelated iron (MS) alone or MS iron with 100 mg (1 ×) or 200 mg (2 ×) of EDDHA chelated iron (sequestrene 138) added per liter. *Data points* are labeled with the treatment and month sampled.

the upper node (data not shown). Individual color values of red, green, and blue were not consistently significant over the treatments and times tested for either node (data not shown). The upper node showed a consistent increase in MNDVI values over time for all treatments (Fig. 2). Differences were not significant for the initial reading and 3 mo. for 'Hallertauer Tradition' if stored on medium with EDTA chelated iron (MS formulation) alone and with 100 mg l^{-1} EDDHA chelated iron (Fig. 2). Since the visual evaluation was done on the whole plant and the aim of this project was to compare the visual evaluation with the image analysis, a consistent value was needed for the comparison. This result differs from our earlier study of pear where the lower node was more indicative of change during storage (Aynalem et al., 2006). In neither case was the mean of the two nodes as useful as the individual node. All of the image data for individual genotypes had a large amount of variation due to the physical nature of the bags used for storage. It is difficult to achieve uniform exposure for the uneven surface that is photographed. This is the likely reason that a ratio-based MNDVI correlates more strongly with visual evaluations than other pixel expressions. Ratio based expressions have also been more useful in other studies where uneven exposure occurs.

Comparison of visual evaluation and image analysis. Most of the cultivars showed positive correlations between MNDVI values for the upper node and visual ratings of the whole plant. Only 'USDA 21119' correlations were below $r^2 = 0.5$ (Fig. 3). The MNDVI values increased over time. The values at 6 mo. storage were generally greater than the 3 mo. ratings. MNDVI values show some genotype variation, with 3 and 6 mo. values clustering slightly below zero for three cultivars and slightly above zero for the other five cultivars (Fig. 3). The positive correlations indicate that image analysis could be applied as an alternative-evaluation technique of evaluation for *in vitro* stored hops.

Iron formulation effects. The overall visual and MNDVI means for all genotypes showed significant differences between the control and the stored plantlets (Fig. 4). Visual ratings for plants on MS iron were significantly better than those on the sequestrene iron at 3 and 6 mo.

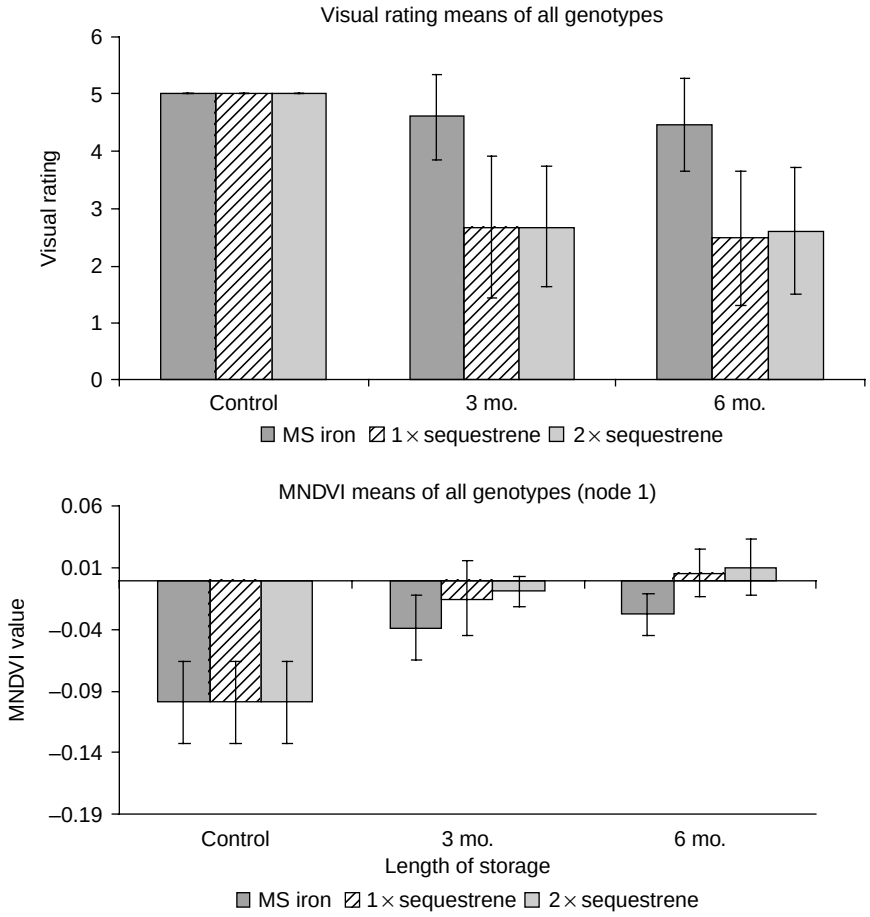

FIG. 4. Mean of visual ratings and modified normalized vegetation index (MNDVI) values from digital photos of *in vitro* cultures of the upper node of eight hop (*Humulus lupulus*) cultivars stored at 4°C for 6 mo. on NCGR-HUM medium with EDTA chelated iron (MS iron) alone or MS iron with 100 mg (1 ×) or 200 mg (2 ×) of EDDHA chelated iron (sequestrene 138) added per liter compared with the control (0 mo.). *Bars* represent means ± SD of five replications (plantlets).

For plantlets stored on EDTA chelated iron (MS formulation) alone when compared with plants on medium with sequestrene iron, MNDVI values were significantly different between the initial reading and 3 or 6 mo. for all hop cultivars. The MNDVI means of all genotypes showed significant differences between MS iron and the 2 × sequestrene treatment at 3 mo. and both sequestrene treatments at 6 mo. (Fig. 4). In sharp contrast to the improved growth of *Humulus* accessions at 25°C, this study showed that adding 100 or 200 mg l^{-1} sequestrene-chelated iron to the storage medium resulted in much shorter storage times than the standard MS EDTA-chelated iron formulation. Iron is an essential mineral for growth and development of plants; however, it is also involved in free-radical mediated oxidative stress (Benson et al., 1995). Secondary oxidative stress manifests as browning, necrosis, and death of tissues exposed to low temperatures (Benson 1990). The improved iron availability that resulted in superior growth of cultures at 25°C was likely also the cause of more rapid decline of the plants in 4°C storage. Additional iron availability may be detrimental to growth of plant tissues at low temperatures due to the pro-oxidant properties of iron.

In conclusion, changes in the health of the plantlets could be observed both visually and through digital image analysis. This study enabled us to verify that the image analysis MNDVI value of the upper node of *in vitro* stored hop plantlets correlated with the standard visual rating system. We demonstrated that image analysis was effective for the evaluation of the health of the *in vitro*-stored plants and also for evaluating the effect of iron formulation on the storage potential of hops. Continued development is needed to determine a common sampling point for all types of plants. Further, development of this technique could be useful for evaluation of *in vitro*-stored cultures.

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