Basal media optimization for the micropropagation and callogenesis of *Cannabis sativa* L.

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Abstract

Micropropagation of *Cannabis sativa* L. is an emerging area of research for genetic storage and large-scale production of clean planting material. However, existing protocols were developed using a limited number of genotypes and are often not reproducible. Previous studies reported MS + 0.5 μM TDZ to be optimal for Cannabis micropropagation, yet in our preliminary studies this medium resulted in excessive callus formation, hyperhydricity, low multiplication, and high mortality rates. Following an initial screen of five basal salt mixtures commonly used for micropropagation (WPM, MS, B5, BABI, and DKW), we determined that DKW produced the healthiest plants. In a second experiment, the multiplication rate and canopy area of explants grown on MS + 0.5 μM TDZ and DKW + 0.5 μM TDZ were compared using five drug-type cultivars to determine if the preference for DKW was genotype-dependent. Four out of five genotypes had significantly higher multiplication rates on DKW + 0.5 μM TDZ with the combined average being 1.5x higher than explants grown on MS + 0.5 μM TDZ. The canopy area was also significantly larger for plants cultured on DKW + 0.5 μM TDZ for four out of five genotypes with the combined average being twice that of explants grown on MS + 0.5 μM TDZ. In the third experiment, callogenesis was compared using a range of 2,4-D concentrations (0-30 μM) on both MS and DKW. Greater callus growth was observed on DKW than on MS. While further improvements are likely possible through media optimization, this study represents an important step towards developing standardized micropropagation practices for Cannabis.

1 Introduction

As the commercial Cannabis industry goes through a rapid period of growth, growers are challenged by a lack of a uniform, true-to-type seed for most commercially desirable cultivars. Instead, commercial cultivation of Cannabis is dependent on vegetative propagation, generally from mother plants, to ensure a consistent crop (Chandra et al., 2017). Maintaining mother plants requires considerable production space (up to 15% of total growth space) while also risking infection of these valuable genetic resources and the subsequent propagules by pests, disease, and viruses. This, combined with the limited number of pesticides registered in the Cannabis industry, makes clean starting material vital for commercial production. Mircopropagation is an alternative approach for vegetative propagation that eliminated many of these risks. With an effective *in vitro* protocol, many genotypes can be maintained with little risk of pest or disease using significantly less space while providing a consistent supply of clean plants (George and Debergh 2008; Chandra et al. 2017).

Early attempts at micropropagation of medicinal and fiber cultivars of Cannabis can be traced back to the 1980s, with these studies assessing the recalcitrance of Cannabis to tissue culture (Richez-Dumanois et al. 1986). Due to prohibition, early studies were limited in number and scope. However, in the last decade Cannabis tissue culture has seen a significant increase in the number of publications addressing many aspects including regeneration, synthetic seed production and screening for elite cultivars (Lata et al. 2009b, 2010; Piunno et al. 2019). Significant challenges still exist in Cannabis research and recent *in vitro* studies using medicinal Cannabis have only assessed a few cultivars, with most developed using a single genotype (Lata et al. 2010; Farag 2014; Movahedi et al. 2015; Piunno et al. 2019).

Contemporary studies of Cannabis tissue culture have relied almost exclusively on media made with Murashige & Skoog basal salts (MS; Murashige and Skoog 1962; Chandra et al. 2009; Lata et al. 2010, 2016; Farag 2014; Movahedi et al. 2015; Chaohua et al. 2016; Piunno 2019; Smýkalová et al. 2019). One of the most successful protocols developed for drug-type Cannabis found that MS supplemented with 0.5 μM thidiazuron (TDZ) was ideal for shoot multiplication (Chandra et al. 2009, 2011; Lata et al. 2009b, 2010; Movahedi et al. 2015; Chaohua et al. 2016; Piunno et al. 2019). Preliminary work conducted in our lab assessed long term cultures of Cannabis on MS media with 0.5 μM TDZ, hereafter referred to as MS-T05. In our hands, cultures grown on MS-T05 were not as healthy and had much lower multiplication rates than reported in the literature, and plant response varied considerably among cultivars tested. Plants grown on MS-T05 exhibited high levels of callusing, hyperhydricity, signs of nutrient deficiency (Figure 1F-H), low multiplication rates, and ultimately plant death in some cultivars. Based on this, we hypothesized that MS basal salt is not ideal for the micropropagation of Cannabis and that an improved medium would increase plant growth to facilitate micropropagation of a wider range of genetics.

To identify an improved medium, a preliminary screen of five basal salts was conducted. These basal salts included: Murashige & Skoog Basal Salts (Murashige and Skoog 1962), Woody Plant Basal Salt Mixture (Lloyd and McCown 1980), Driver and Kuniyuki Walnut Basal Salt Mixture (Driver and Kuniyuki 1984), Gamborg's B-5 Basal Salt Mixture (B5; Gamborg et al. 1968), and BABI Basal Salt Mixture (Greenway et al. 2012). From this screen, it was clear that DKW produced the healthiest explants (Figure 1A-E). To confirm this finding and determine if this was consistent across genotypes, we compared DKW vs. MS across multiple genotypes for overall plant health, growth, and callogenesis.

2 Materials and Methods

2.1 Preliminary Experiment – Basal Salt Screen

2.1.1 Treatments and Cultivars

The MS (M524; Phytotechnology Laboratories, KS, USA), DKW (D190; Phytotechnology Laboratories, KS, USA), BABI (B1471; Phytotechnology Laboratories, KS, USA), B5 (G398; Phytotechnology Laboratories, KS, USA), and WPM (L154; Phytotechnology Laboratories, KS, USA) media all included: 0.7% agar (w/v) (A360-500; Fisher Scientific, NJ, USA), 3% sucrose (w/v), 1 mL/L of B5 vitamins (G219; Phytotechnology Laboratories, KS, USA), and were pH adjusted to 5.7 using 1 mM NaOH. For each treatment, 200 mL of medium was poured into a We-V Box (We Vitro Inc., Guelph, ON) and autoclaved for 20 minutes at 121 °C and 18 PSI.

The initial screen (described below) used Cannabis seeds provided by Canopy Growth Corporation, the seeds were sterilized using 10% bleach (10 min.) and rinsed with sterilized distilled water three times. The second screen used explants from a clonal drug-type *C. sativa* cultivar (BA-1; HEXO Corp., Gatineau, Quebec).

2.1.2 Basal Salt Screen

Initially, the basal salt screen was performed using Cannabis seeds on MS, DKW, and WPM basal media with four boxes per treatment and four seeds per box. The second basal salt screen was performed using two-node explants from the BA-1 cultivar grown on MS, DKW, BABI, and B5 media. There were five boxes per treatment and five explants per box. These explants were taken from plants that were maintained on media containing: DKW (D190; Phytotechnology Laboratories, KS, USA), 1 mL/L PPM (Plant Cell Technology, Washington,

D.C., USA), 0.7% agar (w/v) (A360-500; Fisher Scientific, NJ, USA), 3% sucrose (w/v), and 1 mL/L of B5 vitamins (G219; Phytotechnology Laboratories, KS, USA). Both screens were conducted using a completely randomized design and the boxes were placed on the same shelf within a controlled environment growth chamber at 25 °C under a 16-hour photoperiod. Photosynthetically active radiation (PAR) and light spectral data (Figure 2) were obtained using an Ocean Optics Flame Spectrometer (Ocean Optics, FL, USA). The average PAR of the experimental area was 41 ± 4 μmol s^{-1} m^{-2} (Figure 2). Qualitative observations were taken after one month of growth. Each box was photographed in a photo booth under an incandescent lightbulb with a Canon EOS 70D.

2.2 Comparison Between MS and DKW Media

2.2.1 Treatments and Cultivars

The MS (M524; Phytotechnology Laboratories, KS, USA) and DKW (D190; Phytotechnology Laboratories, KS, USA) media both included: 0.7% agar (w/v) (A360-500; Fisher Scientific, NJ, USA), 3% sucrose (w/v), 1 mL/L of B5 vitamins (G219; Phytotechnology Laboratories, KS, USA), and 0.5 μM TDZ (Fisher Scientific, NJ, USA) and were pH adjusted to 5.7 using 1 mM NaOH. For each treatment, 200 mL of media was poured into a We-V Box (We Vitro Inc., Guelph, ON) and autoclaved for 20 minutes at 121 °C and 18 PSI. The two treatments shall henceforth be referred to as MS-T05 and DKW-T05 respectively.

To evaluate genotypic variability regarding the effect of media, the tested genotypes included cultivars reported to be both sativa and indica and included one male accession. The cultivar codes that were tested were: BA-21 (indica, female), BA-41 (indica, female), BA-49

(sativa, female), BA-61 (indica, male), and BA-71 (sativa, female). These plants were all provided by Canopy Growth Corporation.

The experiment was conducted using a completely randomized design and the boxes were placed on the same shelf within a controlled environment growth chamber under the same conditions as stated previously. The data were collected when the plants were approaching the top of the vessel. Due to differing growth rates among cultivars, observations for BA-21 were taken after 43 days, observations for BA-41 and BA-49 were taken after 41 days, and observations for BA-61 and BA-71 were taken after 35 days.

2.3 Callogenesis Experiments

2.3.1 Preparation of Callus Induction Experiment

Callusing media were prepared with four different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; Fisher Scientific, NJ, USA), either 0, 10, 20 or 30 µM, on each of two basal salts: MS Basal Salt Mixture (M524; PhytoTechnology Laboratories, KS, USA) and DKW Basal Salt Mixture (D190; PhytoTechnology Laboratories, USA). Media consisted of either DKW or MS salts, 3% sucrose (w/v), 1 mL/L PPM (Plant Cell Technology, Washington, D.C., USA), 0.6% agar (w/v) (A360-500; Fisher Scientific, USA) and pH was adjusted to 5.7 using 1 mM NaOH. Media were autoclaved for 20 minutes at 121 °C and 18 PSI. Approximately 25 mL of the autoclaved media were dispensed into sterile 100 x 15 mm Petri dishes (Fisher Scientific, USA) in a laminar flow hood (DFMZ, ON, Canada). Six 1 x 1 cm leaf explants of a clonal, drug-type, *C. sativa* cultivar (BA-1; HEXO Corp., Gatineau, Quebec) were cultured on each petri plate. The Petri dishes were randomly placed on the same shelf within a controlled environment growth chamber under the same conditions as stated previously.

Development of callus on each petri dish was photographed weekly. Weekly photographs were used to measure the surface area of callus produced using ImageJ 1.50i software (National Institute of Mental Health, MD, USA). Callus weight was also obtained 35 days post-induction using an electronic balance (Sartorius Quintix2102-1S; Sartorius, ON).

3 Experimental Design and Data Analysis

3.1 Vegetative Growth Experiments

The experiment comparing MS-T05 and DKW-T05 had three separate trials. The first trial was run with BA-21 using five boxes per treatment and five explants per box. The second trial was run with BA-41 with four boxes per treatment and BA-49 with six boxes per treatment, both cultivars had four explants per box. The third trial was run with BA-61 and BA-71 with five boxes per treatment and five explants per box. The varying box and explant number between trials were due to limited availability of plant material for certain cultivars. For each trial, all explants had two nodes including one apical explant with two visible nodes. These explants were taken from plants that were maintained on media containing: DKW Basal Medium with Vitamins (D2470; Phytotechnology Laboratories, KS, USA), 0.7% agar (w/v) (A360-500; Fisher Scientific, NJ, USA) and 3% sucrose (w/v).

At the end of the trial, each box was photographed in a photo booth under an incandescent lightbulb with a Canon EOS 70D. The images were analyzed using ImageJ 1.50i software (National Institute of Mental Health, MD, USA) to calculate the average canopy area. All statistical analyses were performed using SAS Studio software (SAS Institute Inc., Cary, NC, USA).

For each box, only the surviving explants were used for observations and calculations. The multiplication rate was measured by subculturing each box and counting how many two-node explants were produced from the original four or five explants. The total average for each box was used in statistical analyses. Two-node explants were used instead of single-node explants since preliminary experience found that single-node explants have a lower survival rate.

3.2 Callogenesis Experiments

Each experiment was run in duplicate. All treatments contained six pseudoreplicates per plate. Three Petri dishes per treatment were used in the first experiment, four were used in the second replication. Results from pseudoreplicates were averaged, and means were used in further statistical analyses. All summary statistics and statistical analyses were performed in Prism (v8.3; GraphPad, Inc. La Jolla, CA, USA).

4 Results

4.1 Preliminary Experiment – Basal Salt Screen

In the first basal salt screen, all seedlings were able to grow tall enough to reach the top of their box when grown on MS, DKW, and WPM, however, the seedlings grown on WPM had abnormally pale leaves (Figure 1D). Since the growth of the seedlings did not seem to differ between basal salts, it was hypothesized that the nutrients within the seed helped override some of the effects of the basal salts. For the subsequent basal salt screen, nodal explants from a clonal line were used since they are more reliant on the nutrients within the basal salts. WPM was also removed from the subsequent screening since the pale leaves made it clear that WPM would not be a beneficial basal salt. This second basal salt screen used MS, DKW, BABI, and B5 basal salts. The explants on BABI and B5 showed little to no growth after one month and had a high

mortality rate (Figure 1A, B). Explants grown on DKW had darker and broader leaves, and slightly more growth than the explants grown on MS (Figure 1C, E). Based on these results we hypothesized that DKW could be a better basal salt than MS for growing Cannabis in culture.

4.2 MS-T05 vs. DKW-T05

4.2.1 Canopy Health and Surface Area

While all five cultivars had a numerically larger canopy area on DKW-T05 than on MS-T05, this was only statistically significant for 4/5 of the tested genotypes ($p = < 0.01$). The BA-61 genotype did not have a significant difference in canopy area between the two basal salts despite the numerical difference ($p = 0.21$) (Figure 3A). The average total canopy area of all the genotypes combined for DKW-T05 was significantly greater than MS-T05 at 8.84 cm^2 and 4.50 cm^2 respectively ($p = < 0.0001$). Overall, explants grown on DKW-T05 had a 96.4% increase in canopy area compared to those grown on MS-T05.

It was visually observed across all genotypes that explants grown on DKW-T05 had lower rates of hyperhydricity, darker and broader leaves than explants grown on MS-T05. Leaf morphology on the explants grown on MS-T05 tended to be long, thin, and curled (Figure 1G) while those on DKW-T05 appeared normal and fully expanded. Slight yellowing around the edges of explant leaves was observed in both treatments (Figure 1G-I).

4.2.2 Multiplication Rate

Similar to canopy area, all five cultivars had a numerically greater multiplication rate on DKW-T05 medium, but this was only statistically significant for 4/5 of the tested genotypes ($p = < 0.05$). The BA-61 genotype did not have a significant increase in multiplication between the two basal salts ($p = 0.17$) (Figure 3B). The multiplication rate of all the genotypes combined was

significantly higher on DKW-T05 than on MS-T05, at 2.23 and 1.48 new explants per explant, respectively ($p = <0.0001$). On average, the multiplication rate of explants grown on DKW-T05 was 150.7% of the explants grown on MS-T05.

4.3 Callogenesis

4.3.1 Callus Surface Area

No callus was observed on leaf explants cultured in the absence of 2,4-D over the experimental period on either basal salt (35 days). Callus surface area consistently increased over the experimental period in all treatments of 2,4-D. Qualitative observations showed more vigorous and healthier callus growth in 2,4-D treatments cultured on DKW basal salts compared with the respective MS basal salt treatments (Figure 4). A comparison of linear regression of the treatment averages (10, 20 and 30 μM 2,4-D) showed that DKW basal salts resulted in a significantly ($p<0.0001$) larger callus surface area than growth on MS basal salts (Figure 5B). Similar trends were observed for both trials; however, the magnitude of the callus surface area was consistently lower in the second trial.

4.3.2 Callus Mass

Leaf explants cultured on MS and DKW basal salts in the absence of 2,4-D (control) did not callus. Control tissues began necrosis after 4 weeks and were dead by the end of the experiment after 5 weeks. Callusing was observed at all three treatment levels (10, 20 and 30 μM 2,4-D) on both basal salts tested. An analysis of variance (ANOVA) with a post-hoc student's t-test found that at all three concentrations of 2,4-D, callus mass was significantly greater ($p< 0.05$) on DKW basal salts compared to the same treatment on MS. This trend was observed in both trials. In the first trial, 10 μM concentration of 2,4-D showed the greatest callus

production of all explants grown on DKW basal salts ($p<0.05$; Figure 5A), averaging 358 mg per explant. There was, however, no significant concentration dependence for callus production observed within DKW treatments in the second trial. Absolute callus masses were greater in the first trial compared to the second trial, as was observed in the callus surface area experiments.

5 Discussion

Previous studies have reported that MS medium with 0.5 μM TDZ is suitable for micropropagation of drug-type Cannabis, with an average multiplication rate of 12.6 (Lata et al., 2009a). However, in our study, nodal explants cultured on this medium resulted in a much lower multiplication rate of 1.48 averaged across five genotypes. One possible explanation for this difference is that there is strong genotypic variation in the response of different cultivars, a phenomenon that has clearly been shown in fiber-type cannabis (Ślusarkiewicz-Jarzina et al. 2005; Chaohua et al. 2016). However, while some variability among genotypes was observed in the current study, the degree of variation was moderate (Figure 3) and none of them approached what was reported by Lata et al. (2009a). Recent reports have suggested that some of the research-grade Cannabis supplied by the National Institute on Drug Abuse (NIDA) is genetically more similar to hemp than to most drug-type Cannabis on the market (Schwabe et al. 2019). As such, the Cannabis used by Lata et al. (2009a) may be genetically different from commercially available drug-type cultivars, such as those used in this study. Given that there are multiple reports of plant regeneration in hemp (Ślusarkiewicz-Jarzina et al. 2005; Plawuszewski et al. 2006; Chaohua et al. 2016; Smýkalová et al. 2019), a possible explanation for the discrepancy between our findings and some existing publications is that these genetics may be more amenable to micropropagation.

While genetic differences could explain some of the discrepancies observed in our study compared to Lata et al. (2009a), it is not the only major difference. Micropropagation is generally divided into five stages including: Stage 0: Selection/maintenance of parent plant material, Stage 1: Initiation of cultures, Stage 2: Multiplication of shoots, stage 3: Shoot elongation and rooting, and stage 4: Acclimatization (Murashige 1974; George and Debergh 2008). In most Cannabis micropropagation studies (Lata et al. 2009a; Smýkalová et al. 2019), nodal explants from plants growing in a greenhouse or growth room are initiated in culture and various media are screened to identify the optimal medium for shoot multiplication. These shoots are then transferred to rooting medium to induce root formation and transferred back to the growth facilities. As such, these studies are optimizing the multiplication rate during the initiation phase (stage 1) rather than the multiplication phase (stage 2), while the present study is working with stage 2 explants. From other species, it is well known that optimal conditions during initiation can be different from the optimal conditions for the multiplication phase. Further, it is common to see an initial flush of growth during the initiation phase, followed by slower and more sporadic growth until cultures stabilize (Murashige 1974; Vardja and Vardja 2001).

This difference could contribute to the very different results observed in our study, where we were using *in vitro* nodal explants as source material and working on stage 2 explants while others have been focused on stage 1 micropropagation. The differential response between these two stages could also explain why we observed a progressive decline and often plant death when maintained on a medium that was optimized for stage 1 micropropagation. Ultimately, one of the main advantages of micropropagation is the exponential multiplication of plants through repeated cycles of subculturing during stage 2, while multiplication in stage 1 followed by

immediate rooting provides only a portion of the potential benefits. As such, the optimization of stage 2 micropropagation is critical for commercial application.

The slow decline of plants when cultured on MS suggests that something in the medium is negatively affecting the plants and the stress is accumulating over time. While MS medium is widely used and very effective for many plant species, there are others that do not do well on this specific nutrient mixture (Driver and Kuniyuki, 1984; Gamborg et al., 1968; Lloyd and McCown, 1980; Greenway et al., 2012). This has led to the development of many different basal media developed for specific plants. In the current study, five different basal salts were evaluated in a preliminary screen and included MS (Murashige and Skoog, 1962), DKW (Driver and Kuniyuki, 1984), B5 (Gamborg et al., 1968), BABI (Greenway et al., 2012), and WPM (Lloyd and McCown, 1980). Of these five media, MS and DKW produced the best results, while WPM, B5, and BABI resulted in very poor growth and were not further evaluated. The composition of these media is summarized in detail by Phillips and Garda (2019) but some of the major differences include the amount and relative proportions of various salts and more specifically the amount and type of nitrogen. It is important to note that of these five, MS and DKW contain the highest amount of total salts, with 4.3 g/L and 5.32 g/L respectively, suggesting that Cannabis prefers a nutrient-rich medium.

Between DKW and MS basal medium, DKW was superior and resulted in higher multiplication rates, larger canopy area, and overall healthier looking plants. While plants on MS medium were very hyperhydric and had extremely deformed leaves that often failed to expand, plants cultured on DKW were visibly less hyperhydric with normally developed leaves (Figure 3). However, it is important to note that the symptoms observed in the present study were based on the first subculture from plants maintained on DKW medium. From previous

experience, the symptoms were more severe when plants were maintained on MS for multiple subcultures, and this often resulted in plant death. For example, while the BA-61 genotype did not show any significant difference when grown on DKW-T05 compared to MS-T05, we previously found that maintaining BA-61 on either MS or MS-T05 over multiple subcultures resulted in a decline in explant health and almost killed our entire stock of BA-61. When these plants were transferred to DKW based media, they recovered and have since been sub-cultured many times without a significant decline in health. A similar problem with walnut was the original impetus for developing DKW medium (Driver and Kuniyuki, 1984). While the researchers were initially able to obtain multiple shoot formation from walnut on MS and other basal media, after multiple subcultures the explants shifted to callus production rather than shoot formation and within ten subcultures they were no longer able to produce viable shoots (Driver and Kuniyuki 1984). This is very similar to observations with Cannabis, where MS-T05 facilitated explant growth during initial transfers, but over time we observed increased callusing, poor shoot quality, and in some cases explant death.

In comparison to MS basal salts, DKW has similar ammonium to nitrate ratio but has less total nitrogen (Driver and Kuniyuki 1984; Phillips and Garda 2019). While this may contribute to the difference in plant growth, DKW is also higher in total salt levels and has a significantly different composition. Some major differences to note are that DKW includes much higher levels of sulphur (~7x), calcium (~3x), and Copper (10x). Further research is needed to determine which factors are responsible for the improved growth in Cannabis and how this can be further optimized. While DKW was superior to MS, some explants had yellowing around the edge of their leaves on both media, suggesting that there is still a nutrient imbalance and further optimization may be beneficial. Once the basal medium is fully optimized, it would also be

useful to re-examine the type and levels of plant growth regulators and other factors to further improve this system.

Numerous research groups have also explored the formation of and subsequent development of callus in cannabis, mostly with the goal of plant regeneration (Raharjo et al. 2006; Chandra et al. 2009; Lata et al. 2010; Farag 2014; Movahedi et al. 2015; Chaohua et al. 2016; Piunno et al. 2019). These studies have largely used MS basal salts and to the authors' knowledge, none have explored the use of DKW to produce callus (Richez-Dumanois et al. 1986; Mandolino and Ranalli 1999; Ślusarkiewicz-Jarzina et al. 2005; Raharjo et al. 2006; Chandra et al. 2009; Lata et al. 2009a, 2010; Movahedi et al. 2015; Chaohua et al. 2016; Smýkalová et al. 2019; Piunno et al. 2019). Given that whole plants did not perform well on MS and preferred DKW, these two basal media were evaluated for callogenesis. Similar to whole shoot growth, the surface area of 2,4-D induced callus increased significantly faster on DKW compared to MS over the period of study (35 days; Figure 5B). These findings are further supported by the significantly larger callus mass found at all 2,4-D treatment levels on DKW at the end of the 35-day study period (Figure 5A). This suggests that 2,4-D mediated callogenesis is a product of the interaction between basal salts and PGRs, a link that has not previously been explored in Cannabis. The results of this study suggest that for the development of callus using 2,4-D, DKW basal salts are preferable to MS basal salts. However, given that no regeneration was observed, it is not known if DKW would be beneficial for this application. While most research groups have used MS in the proliferation of callus cultures, DKW and other media should not be overlooked as a potentially better option for callogenesis and plant regeneration. Further work is warranted to determine whether the auxin-DKW interaction leads to greater

callus proliferation in all commonly used auxins, or if this enhanced callogenesis on DKW occurs in an auxin-specific manner, as shown by the response to 2,4-D in this experiment.

6 Conclusion

Based on our research, the previously reported Cannabis growth medium of MS + 0.5 μM TDZ is not optimal for all cultivars in stage 2 micropropagation. Cannabis grown on DKW-T05 displayed fewer physiological abnormalities, better multiplication rates, larger canopy area and broader leaves in four out of five genotypes evaluated, in addition to improved callogenesis. While DKW-T05 is an improvement over MS-T05, the multiplication rate remains relatively low. Further improvements in media composition are likely possible and DKW basal salt medium provides a reasonable starting point for this objective. Continued research will ultimately help to make micropropagation a more effective approach for germplasm maintenance and clean plant production.

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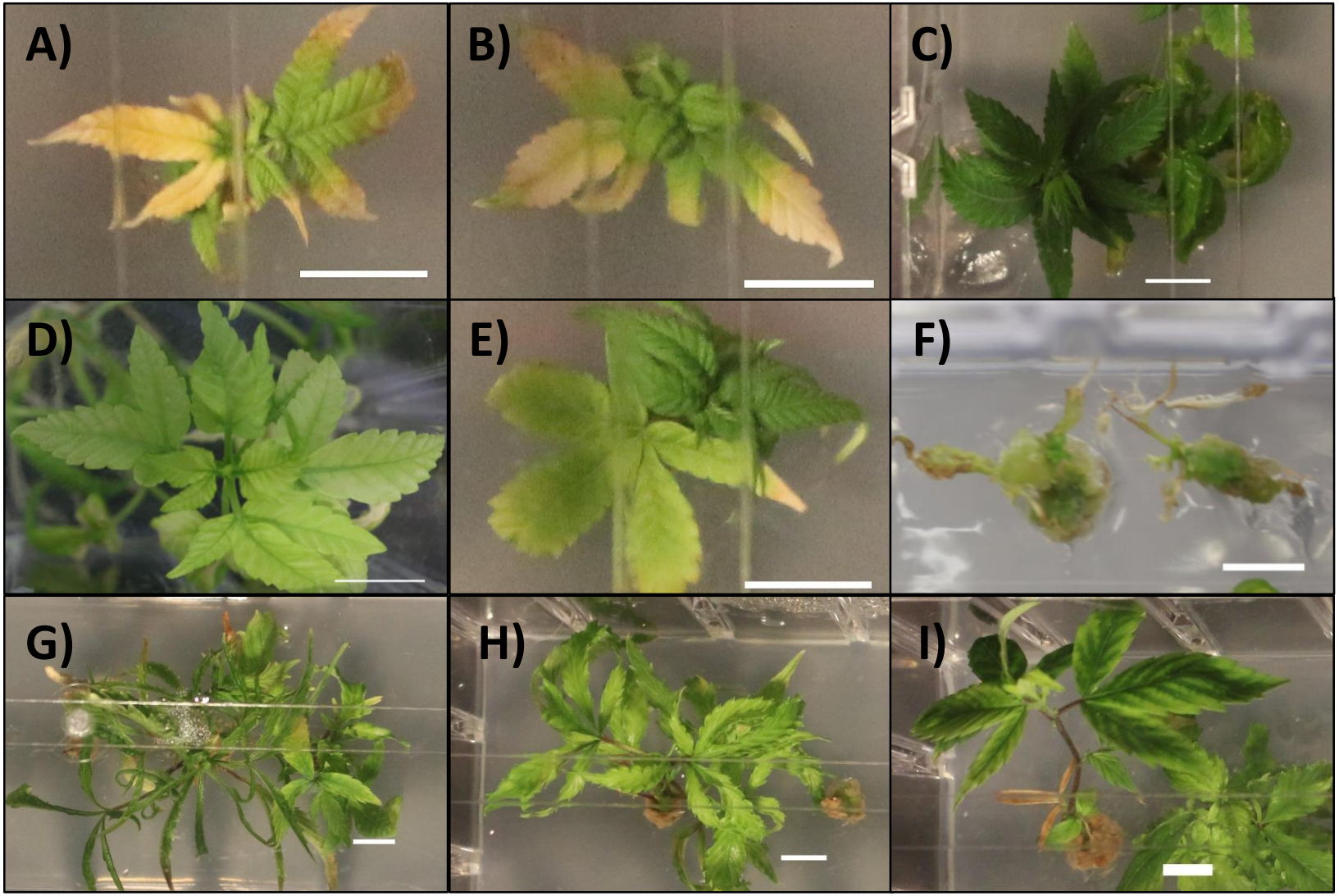

Figure 1: Representative photographs of phenotypic responses to different basal salts. A) B5 basal medium, B) BABI basal medium, C) DKW basal medium, D) Woody basal medium, E) MS basal medium, F) Explant grown on MS + 0.5 µM TDZ with excessive callusing, G) Explant grown on MS + 0.5 µM TDZ with abnormal leaf morphology, H) Explant grown on MS + 0.5 µM TDZ with yellow leaves, I) Explant grown on DKW + 0.5 µM TDZ with yellowing leaves. Scale bar: 1 cm.

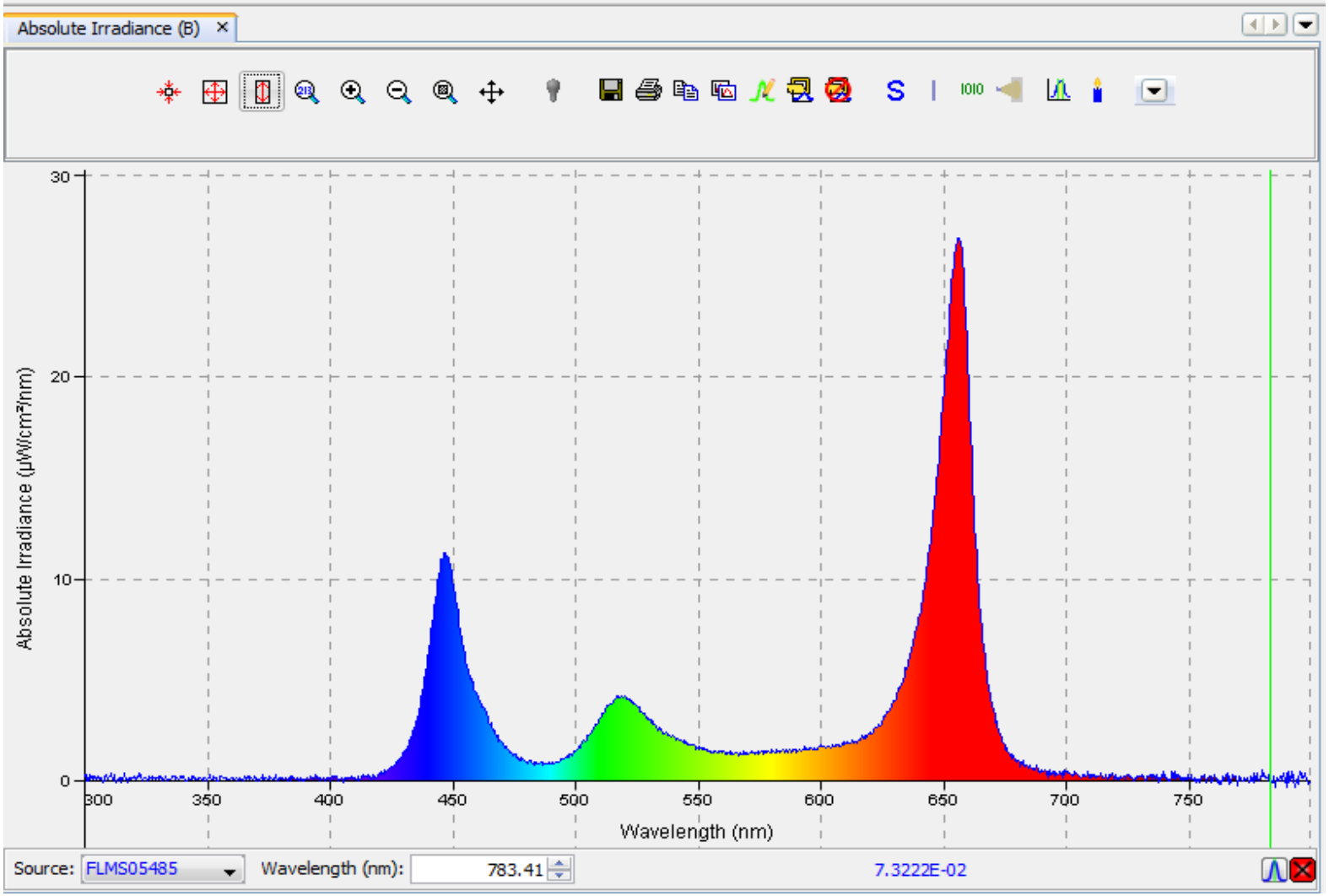

Figure 2: A representative light spectrum of the lighting used in the controlled environment growth chamber. The average photosynthetically active radiation (PAR) over the experimental area was 41 ± 4 µmol s^{-1} m^{-2} using an OceanOptics . Average PAR was calculated using Excel ™.

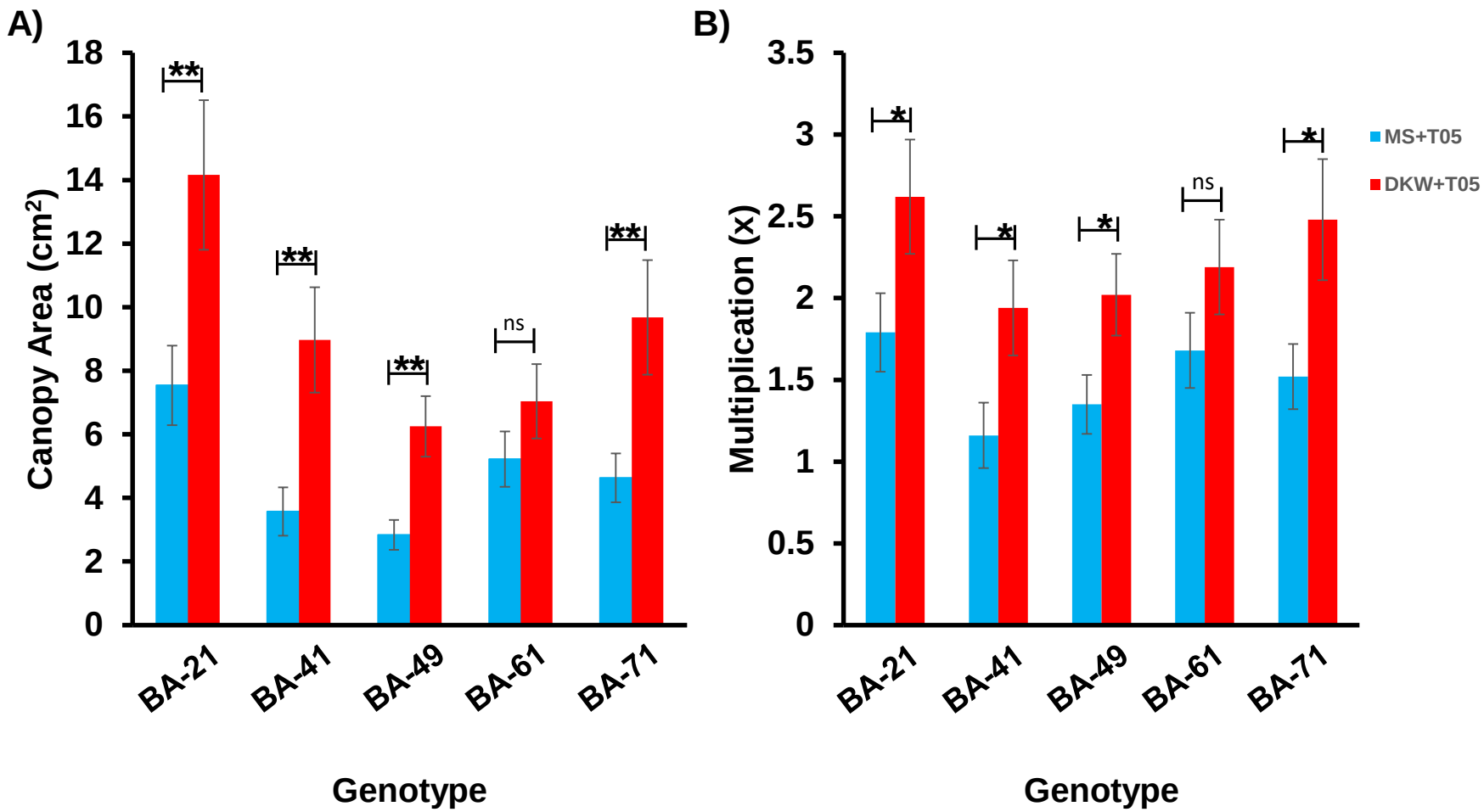

Figure 3: Canopy area and multiplication rate averages from the three trials conducted in this experiment. A) Average canopy area (cm^2) per genotype (ns $p>0.05$; ** $p\leq0.05$; SAS Studio Software). Error bars represent standard error of the mean. B) Average multiplication rate (x) per genotype (ns $p>0.05$; * $p\leq0.01$; SAS Studio Software). Error bars represent standard error of the mean.

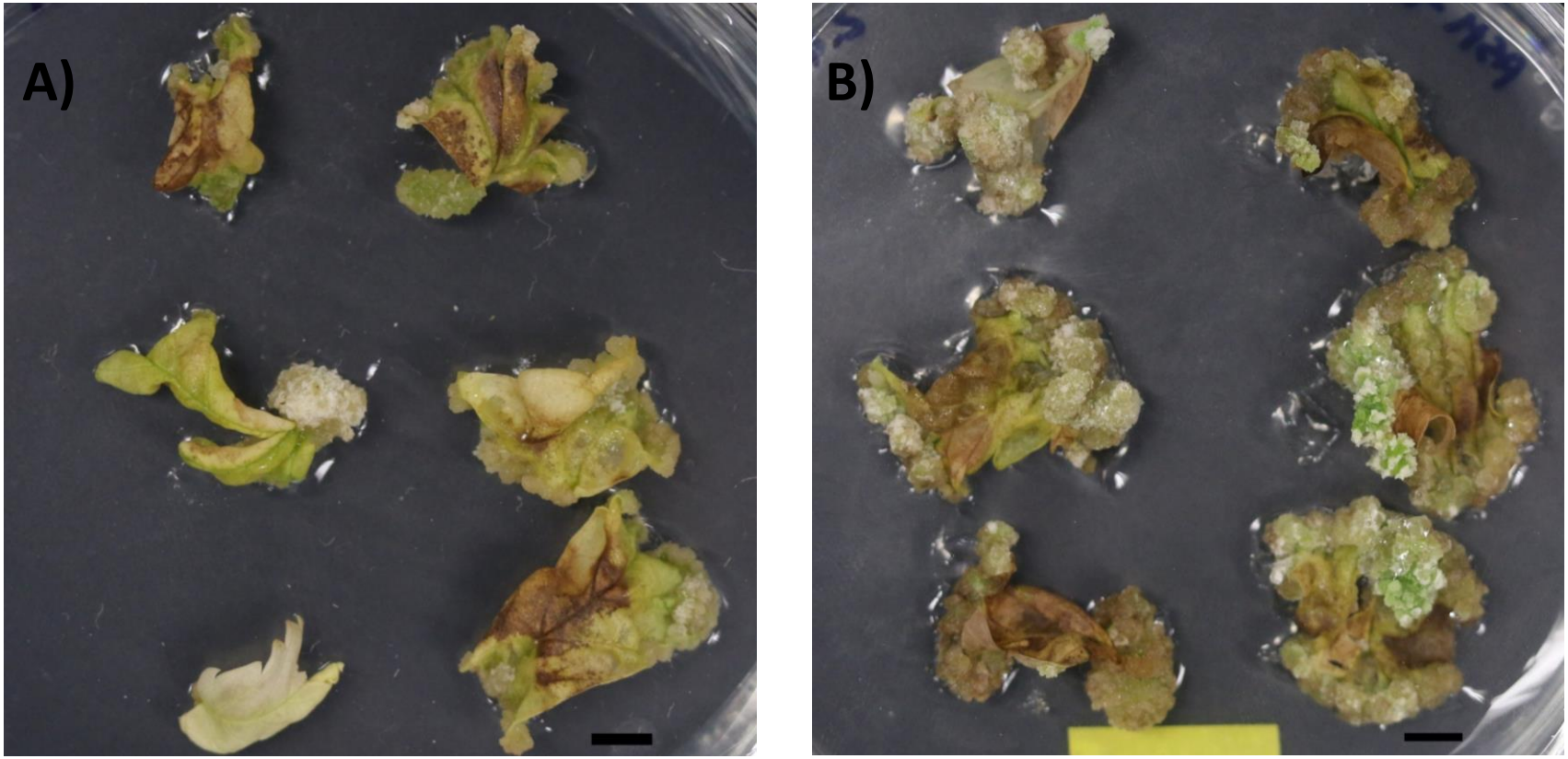

Figure 4: Representative photograph comparing MS+ 10 μM 2,4-D (A) and DKW+ 10 μM 2,4-D (B). Callus growth was more vigorous on DKW media compared to MS media. Scale bar: 5 mm.

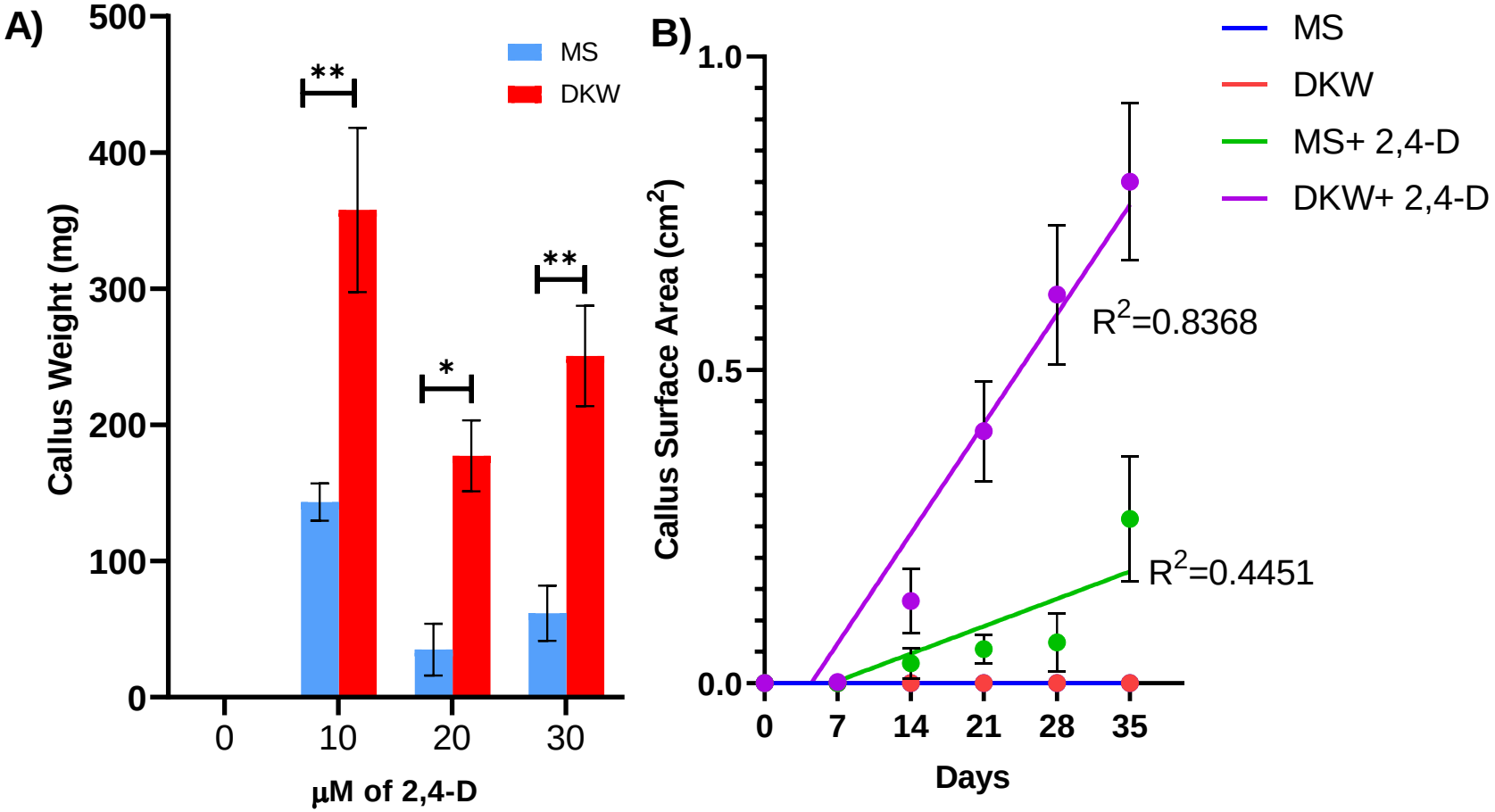

Figure 5: Trial 1 results for callus weight and surface area of leaf disks cultured on MS and DKW media with 2,4-D. Responses in trial 1 and trial 2 showed similar trends, the overall magnitude of response was lower in trial 2. A) Callus weight in milligrams (mg) per leaf disk. Error bars represent standard error of the treatments respectively. * denote significance as determined by a two-way ANOVA with a Tukey's multiple comparison tests (ns $p>0.05$; * $p\leq 0.05$; ** $p\leq 0.01$; Prism v8.3). Similar trends were observed for the second trial. Error bars represent standard error of the mean. B) Linear regression of callus surface area. MS and DKW represent controls with no 2,4-D and did not produce any callus. MS+2,4-D and DKW+2,4-D represent the average surface area responses observed across all 2,4-D treatment levels (10, 20 and 30 µM). Comparison of linear regressions found that the callus response to DKW+ 2,4-D treatments was significantly greater than the MS+ 2,4-D average response ($p<0.001$).