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Optimization of regeneration of red raspberry (*Rubus idaeus* L.) cultivars 'Veten' and 'Ninni' using different light qualities

Emilie Sandell
M.sc. Plant Sciences - Plant Production Systems

Acknowledgements

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Abstract

Customizable light technology is becoming commercially available to producers, giving opportunity to increase control in plant production systems. Understanding of how specific light qualities affect different species and cultivars is necessary to develop light application protocols. In this study, the main objective was to optimize a micropropagation protocol for *Rubus idaeus* (red raspberry) comparing two cultivars, 'Veten' and 'Ninni'. They were propagated through indirect organogenesis. After inoculation of leaf disks and petioles on callus induction media, cultures were subjected to blue, white, red, far red (40 µmol/$s^{-1}m^{-2}$ for 16 h) compared with darkness for 4-6 weeks. During the subsequent shoot proliferation, all cultures treatments subjected also to the other light treatments. Both cultivars responded positively, with variations, regarding explant types and light quality. Blue and red light can give equal or superior results to that of white light for shoot proliferation. Results also indicate high dependency of results on explant type, cultivar and lights quality, which implies that protocols should be customized to cultivars. 'Ninni' had the highest response to varying the explant type and light quality. Further research is needed to investigate applications to regulate morphogenesis and to increase efficiency of micropropagation of *R. idaeus*.

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1. Introduction

1.1. General information on *Rubus idaeus*

Red raspberry (*Rubus idaeus* L.) is a perennial shrub in the Rosaceae family, which bears fruits on biennial stems. Of the more than 400 species making up the *Rubus* genera, *Rubus idaeus* is the most widely distributed. The production spreads across Europe, America and Asia with more than 70 % of the production occurring in Europe (FAO, 2018). It is a sweet, yet tart bramble fruit widely acknowledged as one of the healthiest fruits (Kaume et al., 2012, Simonovic et al., 2019). The contribution of the raspberry fruit to general health and reduction of chronic diseases (Seeram et al., 2006, Zafra-Stone et al., 2007, Chen et al., 2016, Lavefve et al., 2020), in addition to being labeled a "superfood" (Hancock et al., 2007, Boone, 2013), is one of the greatest reasons why in recent years raspberries have gained attention and increased in popularity. As illustrated in Figure 1, world production of raspberries has doubled during the past 20 years from approximately 420 000 metric tons in 2000 to app. 870 000 metric tons in 2018. This has resulted in an increase from 3,7 million metric tons (1998-2008) to 5 million metric tons (2007-2017) of raspberry production in Europe over the past 22 years (FAO, 2018).

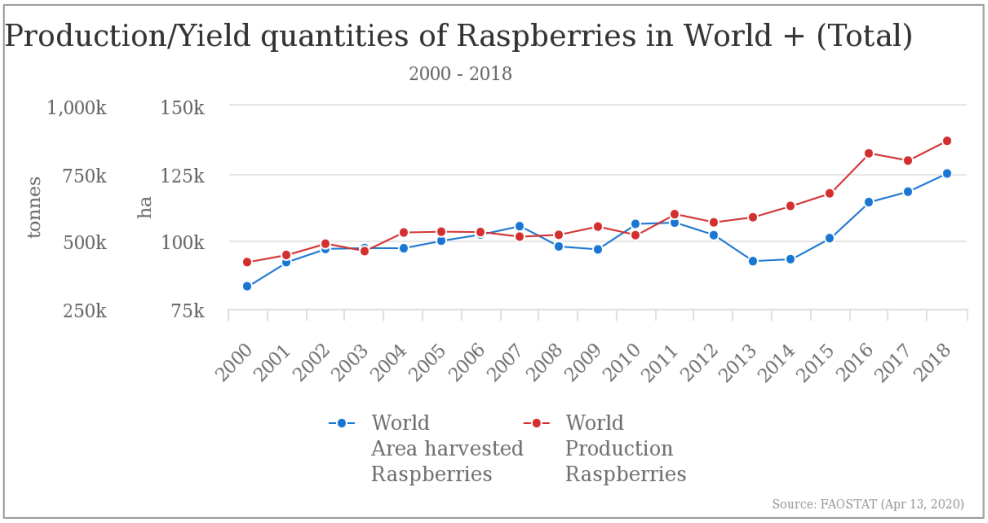


Figure 1: *Total production/yield of raspberry in ha and tonnes from 2000 to 2018 (FAO. 2018. Crops: Raspberry (Visualize) [Online]. Available: http://www.fao.org/faostat/en/#data/QC/visualize [Accessed 13.04.2020].)*

The increase in demand of raspberry can be owed largely to the positioning of berries as functional foods due to their antioxidant activity. A FRAP Assay (Ferric Reducing Antioxidant Power Assay) registered contents of 3,97 mmol antioxidants per 100 g raspberries (Blomhoff, 2002) made up of anthocyanins, phenolics, ellagitannins and vitamin C (Schulz and Chim, 2019, Lavefve et al., 2020). Raspberries are also a source for various nutrients such as vitamin

K, B2, B6 and A, and potassium, magnesium, calcium and iron which all are bioactive compounds which provide essential and/or important health benefits (Bobinaitė et al., 2016, Schulz and Chim, 2019).

Demands of raspberry are increasing every day, creating a need for efficient mass production. With this, new challenges present themselves. The plantlets should be pathogen free and uniform and micropropagation of raspberry presents an appropriate solution.

1.2. The History of Raspberry cultivation

Though the *Rubus* genus has been foraged for millennia, records point to cultivation starting in the 14th century by Turner, an English botanist (Hendrick, 1925) and controlled breeding having its commencement in the 1920s (Graham and Brennan, 2018). The multitude of cultivars that started emerging in the 1980s are derived from hybridization between the European red raspberry (*R. idaeus* L. var *vulgatus*) and the American red raspberry (*R. idaeus* L. var *strigosus*) (Sobczykiewicz, 1984). These cultivars are contained within the two subgenera, Idaeobatus and Eubatus, of which Idaeobatus contains the subspecies *idaeus* and *strigosus* and Eubatus contains the blackberries (Graham and Brennan, 2018).

Due to the vigorous ability of *Rubus* to vegetative spread, it has conventionally been propagated though suckers or root cuttings. After cultivation in a nursery, the propagules are distributed to plantations, where they may continually fruit for 10-15 years. This lends an explanation to the wide geographical distribution of systematic diseases, and thus the high disease pressure in raspberry cultivation (Dolan et al., 2018).

Though much improvement has been made to the compositional and physical quality of raspberries, the focus is now on adaptation to local environments, and disease resistance and susceptibility. Therefore, breeding programs focus on development of quality certified stocks. Maintaining a quality stock, albeit resource and time consuming, creates a foundation of resources for breeding and research, as well as safely multiplying what could be long lived, true to type plant material.

1.3. Micropropagation

Due to challenges such as those disseminated by previously used propagation techniques, optimization of *in vitro* propagation of raspberry has become an important area of research. *In vitro* propagation refers to the aseptic multiplication and regeneration of isolated plant tissue. The purpose of studying a culture in vitro is to improve cultivation systems through increased control of growth factors, and by reducing the complexity of interacting factors. The growth

factors are temperature, relative humidity, light and hormone and nutrient composition. *In situ* these interact and give correlating effects, but *in vitro* they can be studied under complete control.

The purpose of micropropagation, except from producing disease-free plants, is to improve understanding of growth factors, maintain cultivars and to multiply plants that have been genetically modified. Many plants can be produced from a small number of source material, in small spaces.

In addition to the growth factors, the success of micropropagation depends on explant material and the pathway of regeneration. The explant can multiply by acquiring a pluripotent mass called the callus, of which a small portion gives rise to a shoot via *de novo* shoot organogenesis. This is an example of indirect organogenesis (Shin et al., 2020). Direct organogenesis happens as a result of the reprograming of an already present organ (Sugimoto et al., 2019).

In vitro propagation methods for raspberry have been developed over the last 50 years, with the first paper on successful regeneration by Anderson (1980). Raspberry is particularly recalcitrant to micropropagation. There is a multitude of cultivars, and there is great variation between cultivars in their requirements for multiplication and regeneration (Reed, 1990, Zawadzka and Orlikowska, 2006b, Wu et al., 2009). *In vitro* stocks as explants have yielded far higher multiplication rate than greenhouse grown plants (Cousineau and Donnelly, 1991), yet they have thin leaves which desiccate rapidly. Raspberry also has a high production of phenolic compounds in the leaves which accumulate, oxidize and cause browning of the tissue, ultimately lowering the regeneration rate (Benson, 2000). Thus, optimization of micropropagation protocols is needed in order to develop our efficiency in cultivating this crop.

1.4. Light quality as a Growth Factor

Since plants are sessile, they have developed sensory systems to receive signals from their environment. Light is one of the main signals received by plants. The molecules that receive and monitor light are called photoreceptors and are responsible for initiating photoresponses (Chen et al., 2004, Casal and Yanovsky, 2005). The photoreceptors that have been identified are divided into families based on their molecular structure, namely phytochromes, cryptochromes, UV B-photoreceptor and phototropin (Taiz et al., 2015). These receptors are responsible for identifying the quality and quantity of light, in addition to the daylength and direction from which it is irradiated. According to the light a plant receives, the photoreceptors send signals to alter the endogenous hormonal balance.

The phytochromes (PhyA-E) absorb light waves from the red and far red (600-750 nm) part of the electromagnetic spectrum, as well as blue light (350-500 nm) and UV-A (ultraviolet-A) (320-400 nm). When the phytochrome absorbs red light, or is left in the dark, it is converted into its inactive form (P_r). Vice versa, when it absorbs far red light, it is converted back into its active form (P_{fr}) (Rockwell et al., 2006). The different phytochromes are associated to the function of promotion and inhibition of etiolation, seed-germination, stem elongation and flowering. The phototropins are associated with phototropism, stomatal opening and chloroplast movement. Cryptochromes are responsible for many events and developmental patterns (Lin and Shalitin, 2003). Some examples are suppression of stem and petiole elongation, circadian clock entrainment and anthocyanin production. These two families absorb blue light and UV-A (ultraviolet-A) irradiation. UV-A irradiation is also absorbed by ZTL (Zeitlupe), while UV-B (ultraviolet-B) is absorbed by UVR8 (UV RESISTANCE LOCUS 8) (Jenkins, 2014, Sanchez et al., 2020). In orchestra, the photoreceptors initiate signals that influence the plants perception of time and trigger hormonal responses.

LEDs (Light Emitting Diodes) are part or the technology that helps expand the potential of spaces in which plant can thrive, and the control that can be exercised over plant cultures. Though the first LED was developed in the early 1900s, the first wavelength specific LED was developed by Holonyak and Bevacqua (1962). The next important development was commercializing their production to make them available for production purposes. LEDs are not only beneficial because of their significantly higher electrical efficiency (µmol/J) (Barta et al., 1992), but also because they take up less space and are highly customizable.

Light quality treatments have been of interest for the past 30 years but have become more closely studied in recent years after commercialization. However, the response that the light quality triggers varies between species and even cultivars (da Rocha et al., 2013, Poncetta et al., 2017, Rantanen, 2017). Even more so, the information regarding photomorphogenesis in red raspberry is scarce. Literature points to the most productive light quality being that of red light (620-750 nm) (da Rocha et al., 2013), although this is the only case found in which light quality is tested using LEDs as a light source. Erig and Schuch (2005) applied filters under white fluorescent light and found red light to elongate petioles and minimize leaf size severely, although multiplication of shoots was the most productive under green lights. In experiments by Poncetta et al. (2017) LED composites yielded less efficient multiplication of red raspberry in comparison to fluorescent lights, but with higher quality shoots. Pasa et al. (2012) found

survival of propagated shoots of blackberry to be highest under red and blue light, survival rates which gave superior results to those treated with fluorescent lights.

Divided into light quality, quantity and duration, light influences a range of traits of horticultural interest such as plant stature, flowering time and root size. Researchers take advantage of this in order to increase control of plants during production. Morphogenesis in plants as effected by light needs to be better understood. LEDs provide appropriate tools for improving the understanding of morphogenesis (Agarwal and Dutta Gupta, 2016).

1.5. Research objectives and study questions

The aim of this project is to improve the application of artificial lighting in micropropagation of raspberry to make a more sustainable propagation system. In order to make propagation more sustainable, productivity should increase (higher reproductive value per week of production time) and the effects of specific spectral qualities should be understood to increase control over physiology and development. Control should be improved through understanding what factors lead to yield loss. The main objective of this research was to optimize the micropropagation protocol for *R. idaeus* for the new Norwegian cultivar 'Ninni' and compare this with the longstanding cultivar 'Veten', which has been subjected to numerous studies previously. This will be done by multiplication under lights of different spectral qualities. The samples will be exposed to different light qualities and their response will be surveyed and analyzed.

2. Objectives for research

2.1. General objective:

- To optimize a micropropagation protocol for *R. idaeus* independent of cultivar

2.2. Specific objective:

- To multiply and regenerate *R. idaeus* from different types of explants through indirect organogenesis under darkness and red, blue, white and far red light
- To observe differences in regeneration of *R. idaeus* based on light treatments, explant types and cultivar

3. Methodology

3.1. Source material, location and culture conditions

This project was carried out in the Plant Cell Laboratory, of the Department of Plant Science (IPV) located in Plant Cell Laboratory of NMBU in Ås, Norway. The plant material maintained for research purposes were Raspberry (*Rubus idaeus*) genotypes 'Ninni' and 'Veten', provided by Saga Plant AS (Akkerhaugen, Norway). These were subcultured every 4-6 weeks in autoclaved jars containing 80 ml media. The media contained full MS (4,405 g/L Murashige and Skoog salts), 3 % sucrose (VWR Chemicals BDH), 0,8 % plant agar, 36 mg/L FeEDDHA [Ethylenediamine di-2-hydroxyphenyl acetate Ferric], 0,5 mg/L BAP [6-Benzylaminopurine] and 0,1 mg/L IBA [Indole-3-Butyric acid] and pH adjusted to 5,7. The temperature in the room used for maintenance is 20ºC and with a light intensity 28 µmol/$s^{-1}m^{-2}$.

All hormones, supplemental iron, MS media and plant agar were provided by Duchefa Biochemie.

3.2. Media

Multiplication was done on 30 ml of Media 1 in Petri dishes (9 cm diameter by Heger AS, Rjukan, Norway) under aseptic conditions. Concentrations of FeEDDHA, sugar and MS were equal to that of the maintenance media, except from the hormone concentrations of 0,5 mg/L BAP (6-Benzylaminopurine) and 1 mg/L 2,4-D (Dichlorophenoxyacetic acid). The concentration of agar was first set to 0,8 g/L as per the protocol supplied by this laboratory (Miller, 2019), however it was downregulated to 0,6 g/L after 14 days due to desiccation of plant material.

The regeneration media (Media 2) used equal iron-, MS- and sugar-concentration as in previous media, but with 0,7 % agar and adjusted hormone concentrations (0,1 mg/L TDZ [Thidiazuron] and 0,05 mg/L IBA [Indole-2-Butyric Acid]) adjusted according to the protocol. The protocol chosen (Zhang et al., 2017) was based on Zawadzka and Orlikowska (2006a) due to its application to 'Veten', in the paper. Due to the novelty of the cultivar 'Ninni', the same recipe was applied for both cultivars. Plants were regenerated as described in 2.3.2. on autoclaved media in jars (7,5 cm diameter by Moss Glassverk, Moss, Norway).

3.3. Experiments on Light Quality

The experiments are performed in three phases, henceforth referred to as Phase 1, 2 and 3.

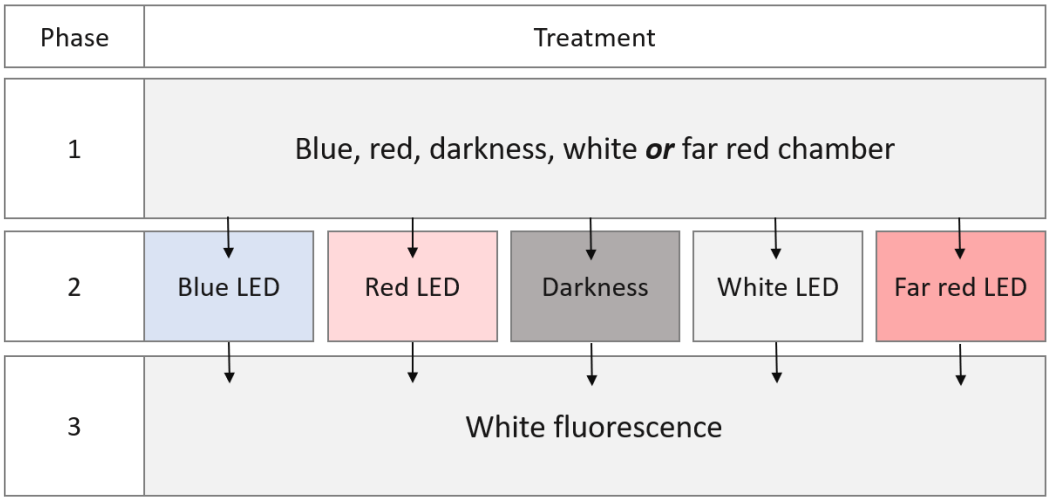
Phase 1 lasts 4-6 weeks and has the aim of multiplication through callus induction on Media 1, with five different light treatments per cultivar.

Phase 2 lasts 4-6 weeks and has the aim of continuing the cultures under the same light as before, and subsequently expose them to all the remaining treatments (see Figure 2) during continued multiplication and shoot induction. The same arrangement is made with Media 1 in Petri dishes as for Media 2 in jars.

Phase 1 and 2 were carried out in a growth room with five chambers with controlled climate conditions. Each chamber contains Philips LED Power Driver 100W/24-V lights. According to previously successful experiments in raspberry maintenance and regeneration, light intensity was set to 40 µmol/m^{-1}/s^{-2} for a 16/8 hour photoperiod and temperature is at a constant 23 ºC (Popescu and Valentina (2000), Zawadzka and Orlikowska (2006b), Lenz et al. (2016)).

Multiplication through indirect organogenesis was done by inoculation of six explants (2.3.2. Experimental variables) onto three 9 cm petri dishes per treatment. For regeneration through shoot proliferation, three samples from Phase 1 were inoculated into jars per treatment. Excess explant material was inoculated when excess Petri dishes and jars were available in order to increase data quantity and to compensate for possible contamination.

Figure 1*: Example of arrangement and treatment of samples during Phase 1-3, showing the subsequent treatment of light of cultures in Phase 2 after Phase 1 light treatments. This is repeated in for the cultures in the remaining chambers during Phase 1 (red, dark, white and far red).*



Phase 3 consists in regeneration in jars of cultures on Media 2 from all treatments under fluorescent light in 23 ºC. This will persist for 4-6 weeks.

The 3 phases are repeated in order to increase the results in Experiment 2 with adjusted duration of the phases. The experiments have 3 variables (light quality, explant type and cultivar) as described in 2.3.2.

3.4. Experimental variables

The following variables were included to test for dependence and to ultimately improve the protocol:

a. Cultivars: ‘Ninni’ or ‘Veten’.

b. Explants. In order to decide what explants were best suited the experiments, three explant types were tested in a small experiment. Petioles, petiole disks and leaf disks (Table 1) were excised with scalpel and cork borer from the healthiest leaves and petioles available. It was quickly decided that petiole disks did not respond nearly as quickly as the leaf disks and petiole, and they were thus removed from the experiment.

Table 1*: Overview of the explant types used for Red Raspberry callus production, the tool by which they were excised and their size. The variation in size of explant is due to variation in mother plant morphology.*

Explant type	Excision tool	Size of explant	Placement
Leaf Disk (LD)	Cork borer	5 mm diameter	Adaxial side against media
Petiole (P)	Scalpel	5-8 mm long	Horizontally, lightly pressed into media
Petiole disk (PD)	Scalpel	2-4 mm wide	Cross section inserted 0,5-1 mm into surface

For consistency and as a result of earlier research by Popescu and Valentina (2000) and Zawadzka and Orlikowska (2006b) the adaxial side of leaves face the media.

c. Light quality: white, blue, red, far red or dark.

d. Container: petri dish or jar.

e. Media concentrations: Media 1 or Media 2 (see 2.2. Media).

3.5. Data Collection and Analysis

Information was registered through continuous stereoscopic imaging. Data was collected through data sheet registrations where type of response is recorded. During Phase 1, data was collected weekly to bi-weekly as callus growth visible to the naked eye. Additional information, such as degree of necrosis, shoots, somatic embryogenesis, crystallization and friable v. compact callus was taken note of during Phase 1, 2 and 3. At the end of Phase 3, the shoots were counted and imaged by digital camera.

Data was analyzed in Statistics Analysis Software (SAS). Due to infections, the data set was not balanced and thus a General Linear Model (GLM) was used. A Proc FREQ Test was run to

test for significance of the following variables: light quality, cultivars and explant types. A significance level of $p<0.001$ was used for all the statistical tests.

Graphic illustrations were created in MiniTab (version 19.2).

4. Results

4.1. Callus proliferation

Due to space limitation, the experiment was performed twice in order to increase data collection. The controls in the experiments which are darkness and white light, were viewed as two of the five treatments and tested statistically as such on their effect on callus proliferation of *R. idaeus*. The significance level in these tests was set to $p<0.001$ to indicate that when p-value is higher than 0.001, the results are insignificant.

The significance level indicates that light quality has a far more significant effect on the cultivar 'Ninni' (p=0.0007) than on 'Veten' (p=0.0123) (Table 2). This effect is summarized in the several significant effects of light quality interaction with explant types on 'Ninni' in Table 3. In this table, there is a significant effect of explant type on 'Ninni' under all treatments. This is far more significant than the effect of explant type under all treatments of 'Veten', apart from the significant response of 'Veten' to explant type under red white light (Table 3).

***Table 2**: Proc FREQ test results for significance of red raspberry (R. idaeus) cultivars (*'Ninni' *and* 'Veten'*) on the effects of light quality. Yes and No represents percentage of incidence or abstinence of callus growth throughout the experiment.*

Row Percent and Frequency of Callus Growth by Chamber and Cultivar								
Chamber	'Veten'				'Ninni'			
	Yes	No	Sum of Samples		Yes	No	Sum of Samples	
Blue	*71.1*	*28.97*	*252*		*74.5*	*25.6*	*270*	
Dark	*55.2*	*44.8*	*192*		*62.98*	*37.1*	*289*	
Far Red	*66.5*	*33.5*	*209*		*76.5*	*23.6*	*276*	
Red	*62.9*	*37.1*	*240*		*72.1*	*27.9*	*258*	
White	*66.1*	*33.9*	*186*		*77.5*	*22.5*	*258*	
P-value	*0.0123*		Total	*1079*	*0.0007*		Total	*1351*

***Table 3:** Proc FREQ test results for statistical significance of starting material (leaf disk and petiole) on callus proliferation of red raspberry (R. idaeus) cultivars* 'Ninni' *and* 'Veten' *under different light qualities (blue, white, far red, red and darkness). Yes and No represents percentage of incidence or abstinence of callus growth throughout the experiment.*

Table of Explant by Cultivar and Chamber					Chi-square test
Cultivar	Treatment	Explant	Yes %	No %	P-value
'Veten'	Blue	Leaf Disk	*67.5*	*32.5*	*0.2114*
		Petiole	*74.6*	*25.4*	
	Dark	Leaf Disk	*52.9*	*47.1*	*0.5012*

		Petiole	*57.8*	*42.2*	
	Far Red	Leaf Disk	*69.5*	*30.7*	*0.4055*
		Petiole	*63.7*	*36.3*	
	Red	Leaf Disk	*55.8*	*44.2*	*0.0079*
		Petiole	*72.6*	*27.45*	
	White	Leaf Disk	*53.7*	*46.3*	*<0.0001*
		Petiole	*83.3*	*16.7*	
'Ninni'	Blue	Leaf Disk	*67.4*	*32.7*	*0.0043*
		Petiole	*82.5*	*17.5*	
	Dark	Leaf Disk	*47.9*	*52.1*	*<0.0001*
		Petiole	*77.9*	*22.1*	
	Far Red	Leaf Disk	*68.8*	*31.3*	*0.0016*
		Petiole	*84.9*	*15.15*	
	Red	Leaf Disk	*61.9*	*37.1*	*0.0007*
		Petiole	*81.6*	*18.3*	
	White	Leaf Disk	*66.7*	*33.3*	*<0.0001*
		Petiole	*88.9*	*11.1*	

The graphic illustrations of the speed of callus growth visible to the naked eye in Figure 3 also indicate that the response is far more dependent on explant type than on cultivar. The rate of fastest to slowest callus growth is interpreted by steepness of the incline of the curve. The order of regressions at y=1 (100 % of samples has grown callus visible to the naked eye) is similar between 'Veten' and 'Ninni', but different between explant types. The steepness of the slope indicates a more rapid acceleration in callus proliferation in petiole cultures than leaf disk cultures.

Looking not at the steepest point of the curves on Figure 3, but at the order of which the regressions reach the reference line (y=1 is when 100 % of the culture is estimated to reach 100 %), a few observations are made. Callus formed from leaf disks are estimated to reach 100% under red and blue light the earliest and nearly simultaneously followed by white, far red and darkness, independent of cultivar. For cultures using petioles as starting material, white light is estimated to be the most rapid in reaching 100 % callus, which is further

affirmed by the significance level of white light on explant types in Table 3. The following light qualities were blue, darkness red and far red.

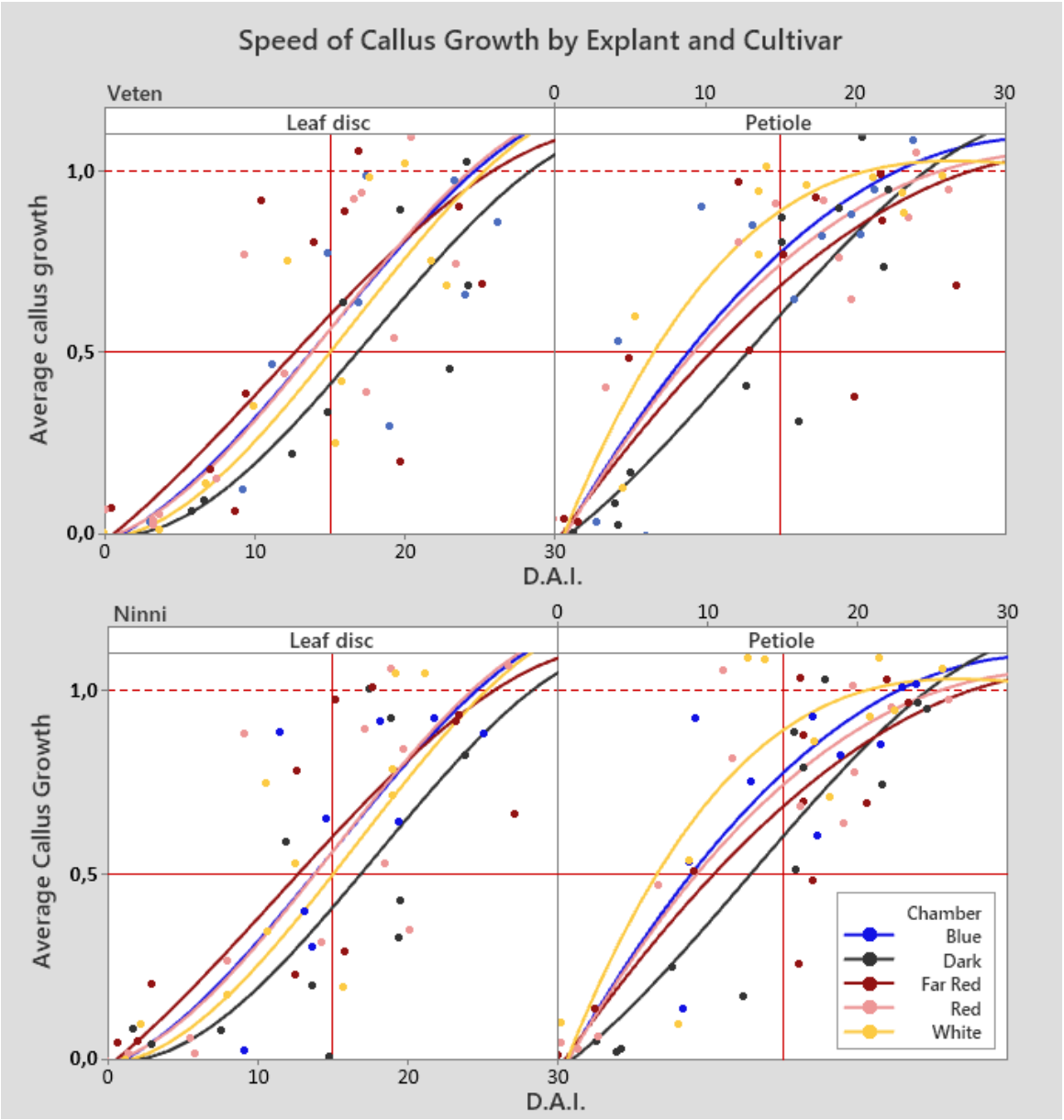


***Figure 3**: The two raspberry cultivars,* 'Ninni' *and* 'Veten' *were inoculated onto multiplication media and given different light treatments (blue, white, far red, red and darkness). The data points represent registrations over time of the average ratio of callus growth (present or not) in each treatment. Reference lines have been inserted at y=1 where callus growth is predicted to have reached 100 % of samples.*

The light quality seems to have a stronger effect on the explant types than on the cultivars, which is affirmed by the highly significant p-value in Table 4. However, it is known from the statistical tests in Table 2 that light quality has a stronger effect on 'Ninni' than on 'Veten', and this is not visible in Figure 3. This points to the importance of testing for statistical significance. Darkness is the least efficient treatment in callus proliferation for leaf disks;

however, it is third to have 100 % of the samples reach callus growth when petioles are the starting material, where far red is the last. The meaning of the peak and inclination of the curves is further interpreted in the discussion.

***Table 4**: Proc FREQ test results for significance of explant type on callus proliferation of red raspberry (R. idaeus) cultivars (*'Ninni' *and* 'Veten'*)*

Explant type	Sum of Samples		
Leaf Disk	*1277*		
Petiole	*1157*		
P-value	*<0.0001*	Total	*2430*

All samples reached 100 % callus in 30 days after inoculation or less, except for contaminated and thus removed samples, and leaf discs of 'Ninni' under red light in experiment 1.

4.2. Shoot proliferation

The crossing of the treatments eventually meant that the amount of treatments increased rapidly, which in turn led to space limitation in the experiments and limited repeats of treatments. Due to the limited amounts of samples per treatment, no significance was detected. More on this in the discussion.

According to the interval diagram of Figure 4, 'Veten' was approximately equally productive independent of explant type, while 'Ninni' was far more productive when explant was petiole rather than leaf disk. 'Ninni' appears to generally be more productive of shoots per sample although the standard deviation is high which suggests great variance in the collected data (Figure 5). This, however, is only reflected in Figure 4 when leaf disks are used as starting material.

ANOVA tests were run in MiniTab to test for the significance (p=0.001) of cultivar, explant type and experiment. None of the tests showed significant results.

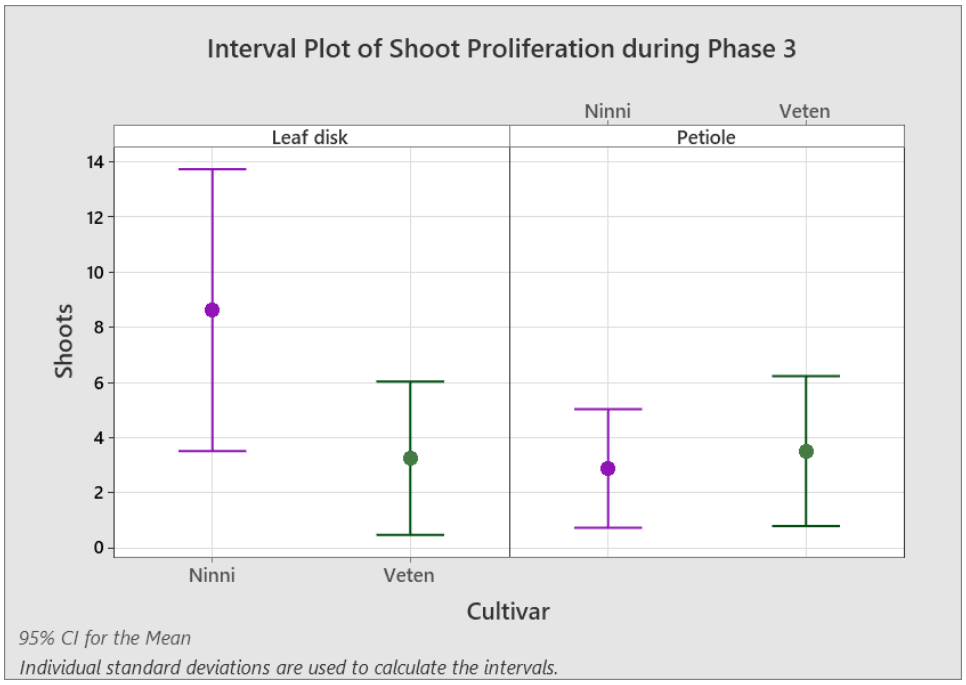


Figure 4: *Interval plot illustrating the average shoot count and its standard deviation of R. idaeus cultivars* 'Ninni' *and* 'Veten'.

As previously mentioned, the two repeats of the experiment are named Experiment 1 and 2. When presented alongside each other, it is apparent that neither cultivars produced significant amounts of shoots in Experiment 2 compared to Experiment 1 (Figure 5). The reasons for this is further examined in the discussion.

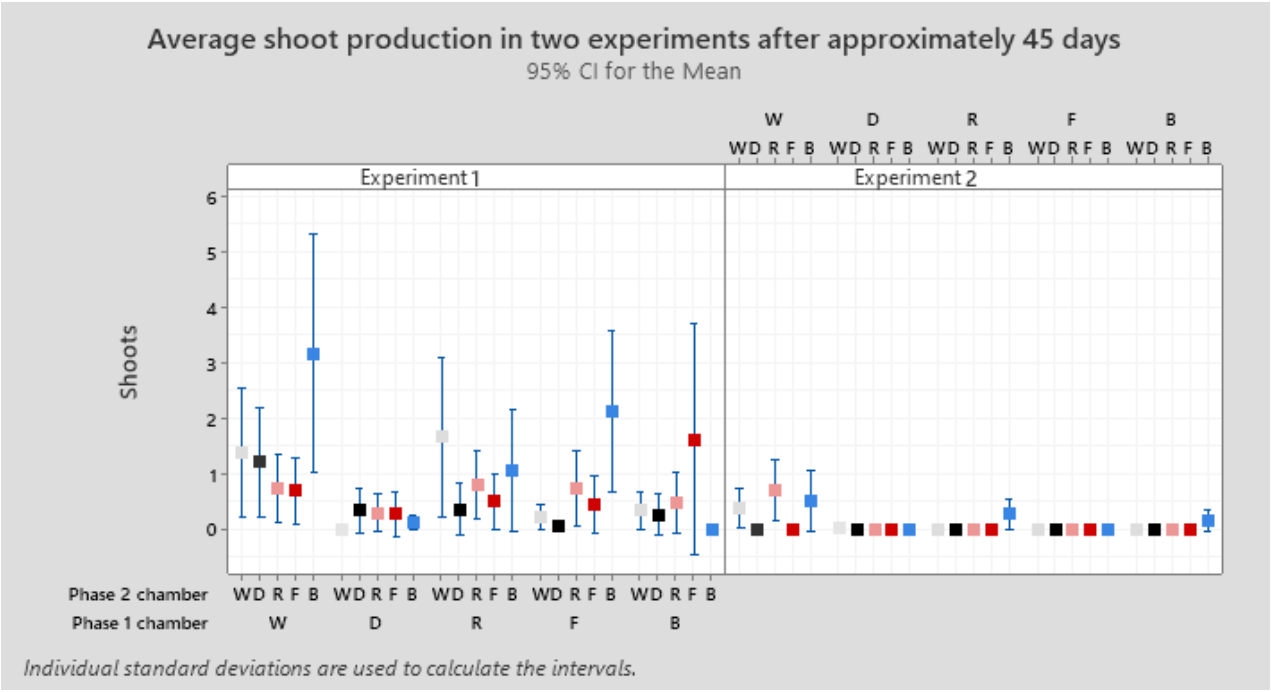


Figure 5: *Interval plot of shoot proliferation of R. idaeus cultivars 'Ninni' and 'Veten' in the two experiments (1 and 2) under light treatments; blue, far red, red, white and darkness. The two graphs illustrate the differences in shoot production between the two experiments.*

The mean shoot count for each treatment of both is calculated for both cultivars and presented in Figure 6. The cultures that continued to proliferate in Phase 2 on fresh callus induction media later went on to produce very few shoots (Figure 6, b) compared to the cultures that were placed on regeneration media in Phase 2 after a period of callus induction (Figure 6, a).

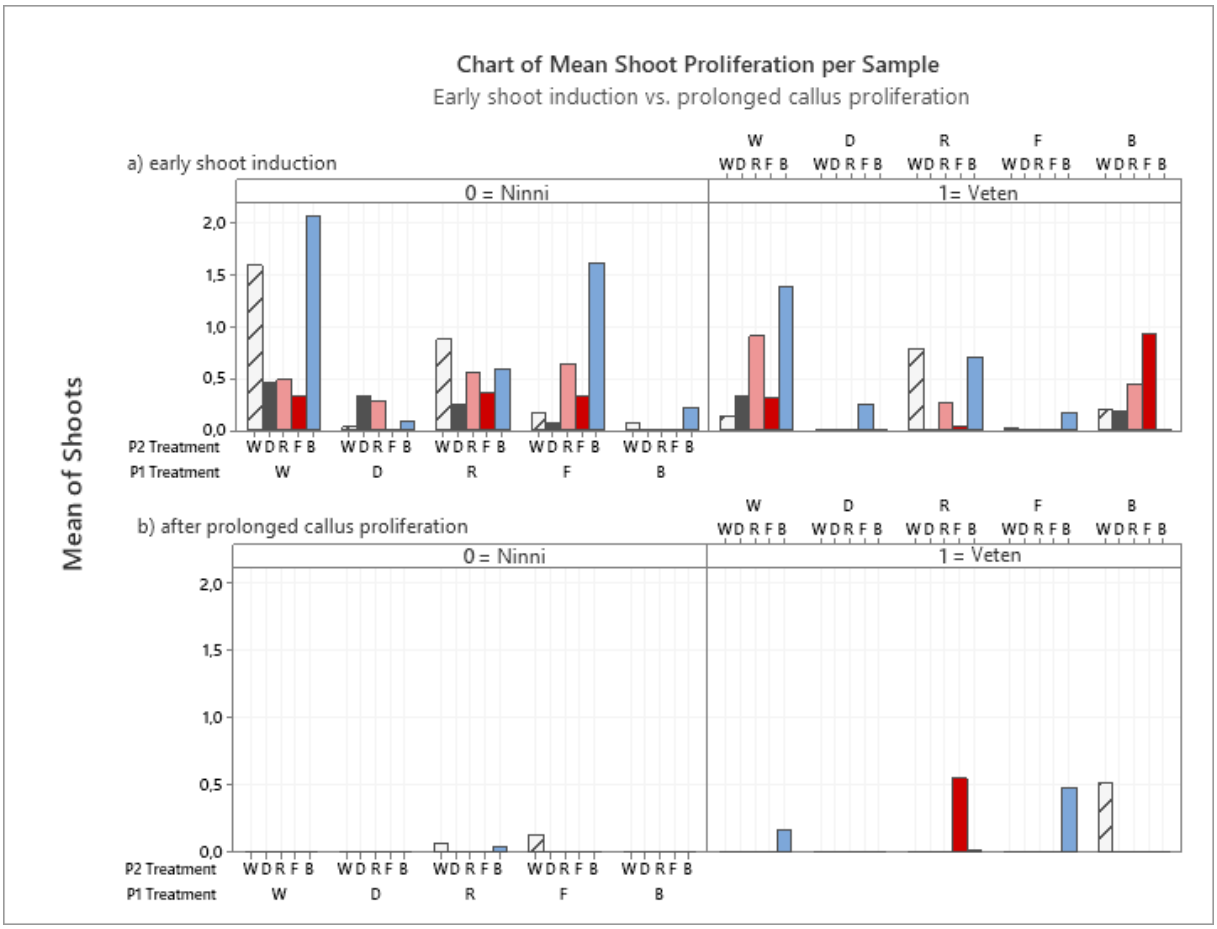


Figure 6: *Bar chart of average shoot count of R. idaeus cultivars* 'Ninni' *and* 'Veten' *in the repeats of the experiment under light treatments; blue, far red, red, white and darkness. The two rows represent the result at the end of the experiments of samples which a) started shoot induction in the 2nd phase of the experiment and b) started shoot induction in the 3rd phase of the experiment.*

4.3. Other observations

A variety of symptoms are thought to have inhibited callus proliferation and thus shoot proliferation. Hyperhydricity is a term used to describe abnormal physical appearance or function, or the formation of crystals of *in vitro* cultured cells (Debergh et al., 1992). For callus cultures, this can either mean a white cast in the cell clusters or a glassy, transparent appearance, as seen in Figure 7, image B. and C. This was very commonly observed in callus cultures obtained from both cultivars and both explant types (Figure 7).

As a response to the stress of being excised from its mother plant, the explants commonly produce phenolic compounds as a response to the stress. Phenolics are secondary metabolites that can lead to the death of tissue. This was also commonly observed both in the days after inoculation (D.) and later in the experiments (F.). Brownish red pigments were also observed in and around callus cultures in later phases of the experiments. This is suspected to be accumulated ferric iron.

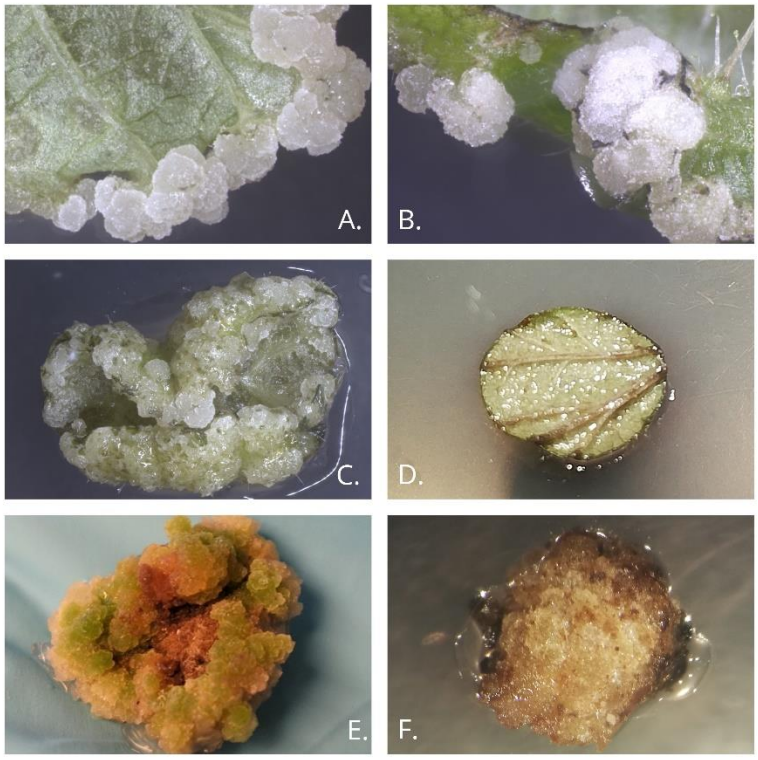


Figure 7: *A variety of results from R. idaeus callus proliferation from leaf discs. Healthy callus clusters (A.), crystal formation in callus (B.), hyperhydricity in callus (C.), early (D.) and late (F.) phenolic production and red discoloration in callus thought to be from iron oxide accumulation (E.).*

For shoots, hyperhydricity can mean glassy, brittle shoots with abnormal growth patterns. Short shoots with underdeveloped leaves as well as meristems that had split into multiple small meristems was observed at the end of both experiments when shoots were examined (Figure 8). This is likely to be a low-quality product although regeneration of these shoots was not attempted.

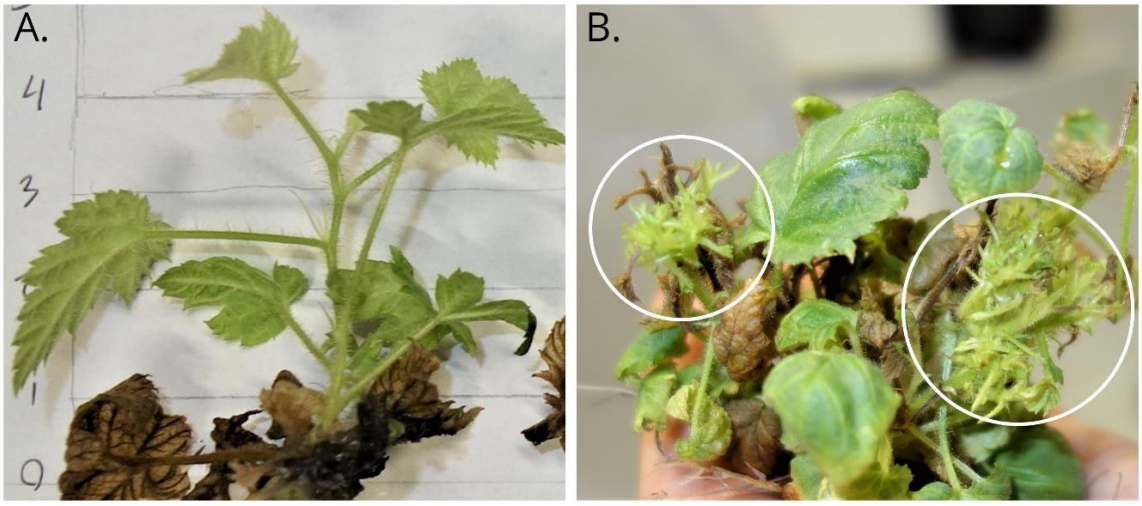


Figure 8: *Expected morphology of shoots of R. idaeus (A.) and symptoms of hyperhydricity appearing as hyperactive splitting of meristems (B.)*

5. Discussion

The main objective of this research was to optimize the micropropagation protocol for *R. idaeus* for the new Norwegian cultivar 'Ninni' and compare this with the longstanding cultivar 'Veten', which has been subjected to numerous studies previously. In the general framework of cell biology and plant science, studying the effects of light quality and intensity is a way to enhance and optimize micropropagation of crops and their control during in vitro phases. This experiment was thus performed and examined as a survey of an existing micropropagation protocol for *R. idaeus* 'Ninni' and 'Veten' with application of red, blue and far red light. The main research question was whether light quality affected callus and shoot proliferation. Additionally, the effects of explant type and cultivar was also investigated. This was done by tracking and imaging callus development after inoculation and by counting samples and shoots at the end of the experiment, followed by graphic and statistical analyses.

Due to known recalcitrance of *R. idaeus* to micropropagation, expectations were that production of phenolic compounds might be in the way of efficient regeneration. Batista et al. (2018) described that cultures kept under light produced more phenols than those kept under darkness.

There are conflicting results for effects of light quality won shoot proliferation in the literature On one hand, some researchers claim that red light might be more efficient than blue light. This is based on previous studies where green (500-600 nm) and red light were the more successful treatments (Yonghua et al., 2005, Erig and Schuch, 2005, da Rocha et al., 2013, Mohamed et al., 2015) for rosacea species such as strawberry and raspberry. Based on this research, the assumption can be made that the higher productivity results of green light are due to the green-yellow side and not the green-blue side of the spectrum of visible light. Other researchers have concluded that blue and white light might be more efficient for shoot proliferation and rooting e.g. in birch (*Betula pendula* Roth) (Sæbø et al., 1995b). This could be explained by of a higher photosynthetic rate when exposed to blue light and the lowest rate when subjected to red light in birch (Sæbø et al., 1995a).

'Veten' and 'Ninni' both have successful micropropagation protocols. However, there are no publications where both varieties share the same protocol. This is the first study optimizing a micropropagation protocol for both commercial cultivars. During regular sub culturing and maintenance, 'Veten' had bigger leaves and larger shoots than 'Ninni'. Zawadzka and Orlikowska (2006b) observed that 'Veten' is more productive relative to a selection of other

cultivars. Expectations were also for leaf discs to be more productive than petioles, due to its frequent use in literature (Popescu and Valentina, 2000, Zawadzka and Orlikowska, 2006a, Lenz et al., 2016). Again, there were no comparison of the two explant types in scientific publications. However, results can be compared to the regeneration protocol used in Phase 2, although it was quite severely altered for the purpose of this research. In the regeneration protocol (Zawadzka and Orlikowska, 2006a) 3,2 shoots were produced per responding explant under white light. As illustrated in Figures 5 and 6, such shoot counts per sample were never recorded in these experiments.

The experiments showed that light quality had a far more significant effect on callus proliferation of 'Ninni' than on 'Veten' (Tables 2 & 3). The strong effects of explant type and light quality on 'Ninni' as compared to 'Veten' could also suggest that 'Veten' is more stable in its response independent of light quality and explant types. This hypothesis is further strengthened by the shoot proliferation balance between the two explant types in Figure 4. The idea that explant type is an important deciding factor in the responses observed is strengthened by several tests and figures presented in this thesis. In Figure 3, speed of callus proliferation is similar between cultivars, but different between cultures started on different explants. Additionally, when testing for significance of explant type, the p-values in Tables 3 and 4 clearly show that explant type strongly affects callus proliferation. For shoot proliferation, this is best illustrated by the different degrees of shoot proliferation (Figure 4) for 'Ninni' between leaf disks and petioles, where the leaf disks are yielding much higher shoot rates than petioles.

The callus cultures kept under blue, white and red light appeared to be the most stressed. This was imaged (Figure 8), but not quantified and hence could not be tested for significance. However, the observation is valid and the callus cultures in darkness and far red light may be healthier at the point of shoot induction. Yet, the highest sum of shoots detected were often found under blue, red and white light according to Figure 6, a). This is an indication that the accumulation of phenols during the early phases is not as harmful as when callus proliferation is prolonged before shoot induction (Figure 6, b). In other words, the age of the callus is more detrimental to shoot proliferation than phenols.

There were no tests conducted on results from Phase 2 and 3 as a result of scarcity in repeats. However, discussing the occurrence and rate of shoots in the final results leads to some interesting possibilities for conclusions. Red and green light has, as mentioned in the introduction, tended to increase productivity albeit occasionally compromising plant health

(Erig and Schuch, 2005, Chen et al., 2019). On the other hand, blue light has been reported to increase plant health through increasing photosynthetic pigments and thus photosynthetic efficiency (Sæbø et al., 1995a) meanwhile occasionally compromising productivity (Fan et al., 2013, Ouzounis et al., 2015). In these experiments, blue light during regeneration certainly posed relevant competition to the other light qualities (Figure 5), although the high shoot proliferation averages came alongside high deviation intervals. It is however remarkable that both red and blue light during Phase 2 yielded shoots in 7/10 of the treatments in this figure, followed by white light which yielded shoot proliferation in 5/10 treatments across the two experiments. It is also notable how samples which callus have been multiplied under white light have had the highest sum of shoot proliferation in both experiments (Figure 5).

There are several unquantified variables that may explain the results of the experiment: the initial state of the explant, the phenolic compounds produced as a stress response (Benson, 2000) and the age of the explants (Hoque and Mansfield, 2004). The leaves which are grown in vitro are small and very thin. In being exposed to air ex vitro, stress is already imposed. Furthermore, using a cork borer to stamp out leaf discs imposes mechanical stress and prolongs the time that they are exposed to air ex vitro. General knowledge is that there are few to no stomata on petioles, which decreases transpiration. Thus, they show fewer signs of stress post-inoculation. However, less wound surface is made on petioles, and this can lead to a slower callus proliferation in the first two weeks (Sugimoto et al., 2019).

How long the plant material has been subcultured at SKP, as well as how long it has been since the last time the mother plants were subcultured may affect the quality of explants and thus their ability to regenerate. The theory that mother plants maintained in vitro have lower regenerative vigor is supported by a number of studies (Mazumdar et al., 2010, Leposavic et al., 2016). The timing of the excision of explants after the last subculturing of the mother plants also effects ability to regenerate. This lends a possible explanation to the vastly different shoot proliferation rate between Experiment 1 and 2 as observed in Figure 5.

Phenolics dissolving into the media and in callus is a common limitation in micropropagation amongst woody plant species (Ahmad et al., 2013). Examples of this is shown in Figure 8 D and F. The suspected inhibition of callus and shoot proliferation due to phenolics indicates that shoot induction should be done far earlier on in the process. Manipulation of callus is causative of stress and thus inhibiting shoot proliferation (Benson, 2000). Furthermore, the age of the callus means an increase in protective substances, such as phenols which has been register to increase after the first fortnight (Ozyigit et al., 2007). It is also relevant to ask

whether iron supplementation is beneficial or not during callus induction, as research by Mukherjee et al. (2013) showed that callus had a high absorption of iron. The supplementation of iron is well reasoned during shoot proliferation to combat leaf chlorosis (Zawadzka and Orlikowska, 2006b), but the addition of iron during callus proliferation might have been detrimental as some explants had stains in the color of iron oxide (Figure 8, E.).

There were many signs of hyperhydricity (Figure 8, B. and C.). Hyperhydricity is often a sign of an excessively humid atmosphere and/or unsuitable media composition (Debergh et al., 1992, Kemat, 2020). For callus cultures, this can either mean a white cast in the cell clusters or a glassy, transparent appearance. For shoots, this often means glassy, brittle shoots with abnormal growth patterns such as extremely short internodes and stems that are broad in diameter. It is generally associated with problems in differentiation (Kevers et al., 2004). Symptoms were observed both during callus and shoot proliferation. This can possibly be explained by excessively humid environments caused by low agar concentration, as previously observed by Kemat (2020). There were challenges with adjusting the level of agar concentration, where the overtly humid environment at 0,6 % agar may have led to hyperhydricity in the callus (Figure 8, E.). On the other side, the concentration from the protocol at 0,8 % led to poor contact between explant and media and hence phenolic production around leaf discs. After testing both concentrations, 0.7 % agar is a reasonable suggestion for callus induction media. Due to difficulty in homogenizing the water and the agar in the making of large quantities of regeneration media, varying degrees of gel strength were observed during inoculation of callus, and this might give rise to unknown variables.

Ultimately, one of the most important limitations of the executed experiments is the experimental design. This led to space limitation in phases 2 and 3, and labor limitation. The lack of understanding of quantifiable data led to time consuming registration of data that ultimately would not give enough samples for statistical analyses. The sum of the different treatments could easily be reduced to increase samples in a few of the treatments which might have given more statistically significant results regarding shoot proliferation in different light qualities. Another possible limitation is confirmation bias, the act of favoring the information that support the expectations. Another is light contamination. In the first experiment, stereoscopic imaging was done often which exposed all samples to fluorescent light. Occasionally during Experiment 1, the samples were exposed to white light for long imaging sessions. This triggered early shoot proliferation for some samples, another possible explanation to the differences between Experiment 1 and 2. The duration of the different

phases varied between Experiment 1 and 2, as observations were made to what could be changed. Whether longer phases (Experiment 1) or shorter phases (Experiment 2) were the best can be discussed, but the high shoot count in Experiment 1, and the lack thereof is not likely to be due to the length of the phases as callus had proliferated sufficiently before inducing shoot proliferation.

There are very few protocols for regeneration by indirect organogenesis in *R. idaeus* since most experiments have used direct organogenesis. Thus it is unclear whether *R. idaeus* regenerates more efficiently via direct or indirect organogenesis (Cousineau and Donnelly, 1991, Turk et al., 1994, Mathews et al., 1995, Mezzetti et al., 1997, Graham and Brennan, 2018).

Suggestions for future research include supplementation of lower amounts wavelength specific irradiation of total irradiation, such as 20-60 %. Literature also shows intriguing results of green light on rosacea cultures (Erig and Schuch, 2005, Mohamed et al., 2015, Poncetta et al., 2017), and adding this to experiments on light quality would be interesting as green light is reported to penetrates lower in the canopy than red and blue light (Bian et al., 2018). Due to the high production of phenolic compounds in *Rubus* and other woody species, shorter light treatments in overall shorter experiments also has potential to improve micropropagation. An adsorbent in the media or pre-treatment to the raspberry leaves could help improve viability of callus and shoot production regarding the phenolic compound buildup. Also, since raspberry is considered a species with health promoting properties, it is a candidate for producing valuable bioactive compounds (Sánchez-Ramos et al., 2018, Bilodeau et al., 2019, Lavefve et al., 2020).

The findings of this thesis imply that the effects of light quality strongly depend on explant type and cultivar, and that there are compositions of light that can compete with the application of white light to micropropagation of *R. idaeus*. In conclusion, although the different light qualities can be beneficial for specific purposes, such as increasing dry matter, chlorophyll content or modifying morphology, composites of different light qualities tend to do better for increasing productivity (Kim et al., 2006, Hung et al., 2016, Bantis et al., 2019, Xu et al., 2020), especially the combination of R and B (Fan et al., 2013). Green light penetrates lower down in the canopy and should for that reason be included in the composition of light for in vitro cultures (Kim et al., 2006, Bian et al., 2018).

6. Conclusion and Future Perspectives

This study shows that efficiency of callus and shoot multiplication can be similar or increased to that of white light by modifying the composition of light quality. It emphasized the relationship between darkness, red, blue, far red and white light and callus and shoot proliferation. Callus proliferation of 'Ninni' was affected by light quality, more so than callus proliferation of 'Veten'. Between the treatments that were tested, blue and red light showed the highest potential for shoot production purposes. Tests indicate that the regenerative abilities of *R. idaeus* is strongly dependent on explant type and cultivar. Therefore, regeneration protocols should be customized to cultivars to save resources. Anyhow, research is needed to improve the understanding of how wavelength specific lights can be applied to optimize micropropagation protocols. From literature and these results, suggestions can be made that future research should investigate composites of red, blue and green lights as sole sources of energy, or supplementations of these light qualities to white light. It would also be interesting to see if *R. idaeus* is a potential candidate for production of valuable bioactive components.

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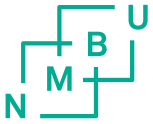

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway