Jaanika Edesi

THE EFFECT OF LIGHT SPECTRAL QUALITY ON CRYOPRESERVATION SUCCESS OF POTATO (SOLANUM TUBEROsum L.) SHOOT TIPS IN VITRO
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Abstract

Cryopreservation enables storage of genetic resources at ultra-low temperatures (<-150°C) while maintaining viability and regeneration capability. The method is especially suitable for long-term preservation of plant materials that cannot be stored as seeds. The genetic resources of potato (Solanum tuberosum L.), one of the most important food crops in the world, are preserved almost entirely by vegetative preservation. Cryopreservation is therefore increasingly applied for securing potato genetic resources in plant genebanks. A major challenge is, however, that recovery percentages can extensively vary among different genotypes.

Light spectral quality is among the most important factors affecting plant growth and morphogenesis, but its effect with regard to cryopreservation has not been studied. In the present thesis, I studied the effect of six different light qualities on cryopreservation success of five potato cultivars before and after cryopreservation. I also explored how the different light conditions affect gene transcript abundance of recovering potato shoot tips.

The results indicate that light spectral quality significantly affects the cryopreservation success of potato shoot tips in vitro. Prior to cryopreservation, cultivation under blue LEDs resulted in high initial survival, while post-cryopreservation exposure to a combination of red and blue LEDs (90% red, 10% blue) doubled the regeneration percentages. Concurrently, for most cultivars, red LEDs had adverse effects both before and after cryopreservation.

The transcriptome analysis of potato shoot tips revealed the complex and extensive effect of cryopreservation on transcript abundance. Moreover, the expression level of stress- and defence-responsive genes was affected by light spectral quality. The positive effect of red-blue LEDs on shoot formation could tentatively be associated with a higher level of morphogenesis-related transcripts and lower level of stress and defence-responsive transcripts. The present thesis reveals that light spectral quality is an additional non-cryogenic factor, which can significantly increase the cryopreservation efficiency of plant germplasm.

Keywords: clonal crop, gene expression, illumination, in vitro, LEDs, long-term preservation, plant genetic resources, transcriptome, vegetative propagation
Edesi, Jaanika, Valon laadun vaikutus perunan (Solanum tuberosum L.) versenkkärien selviytymiseen syväjäädytyksestä in vitro -olosuhteissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Luonnontieteellinen tiedekunta

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Tiivistelmä


Asiassaan: geeniekspressio, in vitro -kasvatus, kasvigeenivarat, kasvullinen lisäys, LED-valot, pitkäkaikaisääilytyys, transkriptomi, valaistus
To my family
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After completing the MSc studies, I remember myself standing in front of the campus restaurant Julinia saying that I would consider doing PhD only if it would rise from future job and the subject of the thesis would have a practical link. One year later, after working in potato genebank at Plant Biotechnology Department EVIKA in Estonian Crop Research Institute (ECRI), I was back in Julinia again but as a novice PhD student with the aim to improve long-term storage of potato genetic resources. Finishing this fascinating project was possible only with the help of many people whom I wish to express my sincere gratitude.

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Abbreviations

“+” cryopreserved
“−” non-cryopreserved control
AFLP amplified fragment length polymorphism
APX ascorbate peroxidase
B blue LEDs
CAT catalase
cv/s cultivar/s
CW cool white fluorescent tubes
D dark
DEGs differentially expressed genes
DMSO dimethyl sulfoxide
GA3 gibberellic acid
GO gene ontology
GPX guiacol peroxidase
GR glutathione reductase
HQI hydrargyrum quartz iodide (Osram), metal halide lamps
IAA indole-3-acetic acid
LEDs light emitting diodes
LN liquid nitrogen
PAL phenylalanine ammonia-lyase
PAR photosynthetically active radiation
POX peroxidase
PPF photosynthetic photon flux
PPM plant preservative mixture
PVS plant vitrification solution
R red LEDs
RB combination of red and blue LEDs
RBF combination of red and blue LEDs with addition of far-red
RIN RNA integrity number
ROS reactive oxygen species
SOD superoxide dismutase
SSR simple sequence repeat
W white LEDs
Original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:


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1 Introduction

Food, feed, fibre, medicine, herbs, spices, wood and other raw materials are just a few examples of plant genetic resources that humankind relies on. Plant genetic resources for food and agriculture, including cultivated crop species and their wild relatives, modern cultivars, landraces, breeding lines and genetic stocks with present or potential value are a foundation for food security (Esquinas-Alcázar 2005; FAO 2009).

Modern crop species and their numerous varieties are a result of thousands of years of domestication based on cultivation and selection of individuals with the most desirable traits (Doebley et al. 2006; Larson et al. 2014). This has resulted in numerous crop varieties, which are adapted to different climate and growth conditions and have good gustatory, cooking and processing characteristics. Modern agriculture favours large-scale cultivation of the selected top-quality varieties with the most economical properties like high yield and low production cost (Esquinas-Alcázar 2005). However, growing homogenous cultivars on large areas increases the risk of susceptibility to new emerging pests and diseases (Hodgkin 2002; Haussmann et al. 2004; Oerke 2006). Moreover, the changing climate conditions set a need for crop varieties, which are adapted to more severe and adverse weather conditions (Haussmann et al. 2004; Chakraborty & Newton 2011). Old indigenous varieties, landraces and their wild relatives, along with present cultivars, provide a reservoir of genetic diversity, which forms the basis for developing cultivars with improved abiotic and biotic stress tolerance, high yield and nutritional value to ensure future food security (Esquinas-Alcázar 2005; Frison et al. 2011; Hufler & Lefeber 2011; Roa et al. 2016).

The importance of preservation of plant genetic resources is recognised worldwide and regulated at national and international levels (FAO 2009; Dulloo et al. 2010; Hufler & Lefeber 2011; Roa et al. 2016). Plant genebanks collect, store and backup a broad range of plant genetic resources with the aim of long-term conservation and availability for breeders, researchers and other users (Dulloo et al. 2010; Hufler & Lefeber 2011; FAO 2014). Currently, over 1750 genebanks around the world hold approximately 7.4 million accessions (FAO 2010). The most feasible storage method is by seeds in dry conditions at a low temperature, which is suitable for many important crop (e.g. maize, wheat, rice) varieties (Li & Pritchard 2009; Pence 2011; FAO 2014). However, the cultivars of several economically important species (e.g. potato, banana, sweet potato) can be propagated only vegetatively, e.g. by stem cuttings, grafts, bulbs, stolons or tubers.
in the field or by in vitro techniques. The long-term storage of these species is more complicated and costly due to the frequent propagation and maintenance activities (Pence 2011).

Traditionally, vegetative crops are stored in field collections (short-term conservation), which is the most common and easiest, but most vulnerable to adverse climatic conditions as well as pests and diseases. In vitro preservation, growing plant material aseptically in controlled growth conditions on artificial nutrient medium, provides a safer means of storage (medium-term), but is more expensive because of the need for specific tissue culture facilities and laborious maintenance of the cultures. For the long-term conservation, cryopreservation, the storage of plant genetic resources (germplasm) at an ultra-low temperature, usually at the temperature of liquid nitrogen (-196°C) or in its vapour (<-150°C), is used. Cryopreservation maintains the viability and potential for resuming plant growth after thawing and is the most secure and cost-effective choice for clonally propagated species (González-Benito et al. 2004; Li & Pritchard 2009; Dulloo et al. 2010). At ultra-low temperatures, the metabolic processes are arrested and no other maintenance activities but ensuring the low temperature are required, and therefore the method is applicable for long-term storage (FAO 2014). For these reasons, cryopreservation is increasingly applied for preservation and backup of plant species/varieties, that cannot be stored as seeds (Jenderek & Reed 2017). However, the main challenge is that each species and cultivar may require optimisation of the cryopreservation protocol before sufficient recovery is achieved (FAO 2014). The minimum acceptable viability percentages vary in different plant cryobanks from 10% to 75% depending on plant material (Volk et al. 2017). The recovery percentages may vary even between the different genotypes of the same species (Keller et al. 2016; Vollmer et al. 2016).

In addition to a cryopreservation protocol (cryoprotection, cooling and rewarming), non-cryogenic factors (e.g. plant health status, pre- and post-cryopreservation culture conditions, in vitro age) affect cryopreservation success of plant germplasm (Reed et al. 2004; Harding et al. 2009). Light intensity is known to affect cryopreservation outcome but the effect of light quality – the composition of different wavelengths – has not been studied before. In general, light quality is among the most important factors affecting plant growth and morphogenesis (Fankhauser & Chory 1997; Devlin et al. 2007). Therefore, in the present thesis, I have studied how light spectral quality during pre- and post-cryopreservation culture affects the cryopreservation success of potato shoot tips in vitro. Moreover, with the help of the available potato genome information (The Potato Genome
Sequencing Consortium 2011; Sharma et al. 2013; Gálvez et al. 2017), I wished to better understand the biological processes behind responses to light quality and cryopreservation. To date, the effect of light spectral quality with regard to cryopreservation has not been studied, and the overall information on functional biological processes related to cryopreservation are scarce. Therefore, the present thesis will shed light on the completely unstudied non-cryogenic aspect, which may significantly affect the cryopreservation success of plant germplasm.

1.1 Potato

1.1.1 Potato as a worldwide food crop

Potato (Solanum tuberosum L.) is the fourth most important food crop in the world after maize, rice and wheat (FAO 2017). Cultivated potato originates from South America but is now grown and consumed worldwide (Hawkes 1990). That is not surprising because potato has high nutritional value with balanced vitamin and mineral content and high yield resulting in usage for food, feed, medicine (Van De Velde et al. 2011), processed products (e.g. chips, alcoholic beverages), as well as raw material for industry (e.g. paper and bioplastic industry) (Gonzalez-Gutierrez et al. 2010). The fact that potato produces much more dry matter and protein per land area with less water usage than cereal food crops makes it attractive for countries where water availability is a limiting factor in agriculture (McGregor 2007). While formerly the biggest potato producers were Russia and Canada in Northern Hemisphere, now the leading producer in the world is China (FAO 2017).

1.1.2 Cultivated potato

Potato belongs to the family Solanaceae, including many other economically important crops, like tomato (Lycopersicon esculentum), eggplant (S. melongena), peppers (Capsicum) and tobacco (Nicotiana tabacum). The genus Solanum is highly diverse with more than 1000 species. A part from these are the tuber-bearing species (subsection Potate in section Petota), which are highly diverse and adapted to different growth conditions through South America up to south-west North America (Hawkes 1990). Historically, tuber-bearing species have been divided into more than 200 species (228 according to Hawkes 1990). The more recent classifications have reduced the number by half, and studies by molecular
methods (AFLP, SSR, barcoding) indicate that the number might still be over-estimated (Jacobs et al. 2008; Jacobs et al. 2011; Spooner et al. 2014). This is due to the complex nature of these species, as they hybridize easily, have a great phenotypic plasticity in different environments, are auto- and allopolyploids, and have both sexual and asexual modes of reproduction (Ovchinnikova et al. 2011; Machida-Hirano 2015).

The economically important potato cultivars belong to the single species *Solanum tuberosum*, which has two subspecies, *S. tuberosum* ssp. *tuberosum* and *S. tuberosum* ssp. *andigenum*. The common potato cultivars (more than 4000) belong to *S. tuberosum* ssp. *tuberosum* (Hawkes 1990). Due to its origin in southern Chile, it is adapted to long daylength, enabling production in northern areas outside of South America. The *S. tuberosum* ssp. *andigenum* and other cultivated species (4 to 7 according to different authors) are mainly cultivated near the Equator mostly in the high Andes from central Peru to central Bolivia, but they can also be found in other areas from South America to south-west North America (Hawkes 1990; Ovchinnikova et al. 2011).

The cultivated potato is highly susceptible to numerous pests and diseases (Hawkes 1990; Machida-Hirano 2015). The Irish potato famine which caused death to over a million people is an example of the worst scenarios that can happen with the emergence of new pests and diseases (Hodgkin 2002). The indigenous cultivars and landraces might lag behind in several agronomic characteristics valued in modern agriculture, but they may present greater disease and pest resistance as well as adaptation to extreme environmental conditions. The Andean *S. tuberosum* ssp. *andigenum* varieties and landraces along with other cultivated species have tubers with highly diverse shapes, colours and patterning. Wild potato species have high alkaloid contents and are therefore not suitable for human consumption *per se*, but they are known to have high disease resistance and adaptation to extreme weather conditions (Hawkes 1990). Therefore, wild species and indigenous landraces form a rich source of genetic diversity that can be utilized for maintaining and improving the disease resistance, adaptability to various climatic conditions, as well as favourable agronomic traits, e.g. tuber shape, nutrition value, vitamin and mineral content of the present and future cultivars.
1.1.3 The challenges related to preservation of potato genetic resources

The diversity of wild potato species and highly variable landraces in the Andean area can be stored by botanical seeds and/or vegetatively as tubers. However, cultivated potato (Solanum tuberosum L.) is a tetraploid with highly heterozygous seeds, and therefore, the genotype can be maintained only by vegetative propagation either by tubers or by *in vitro* techniques (Fig. 1).

Preservation by tubers in field collections is the most feasible but also vulnerable method, for instance due to various pests and diseases, as well as unfavourable weather conditions (Dulloo et al. 2010). Field collections are suitable for short-term storage when varieties are frequently needed (Benson et al. 2011). The *in vitro* technique where the cultures are maintained aseptically under controlled light and temperature conditions on artificial growth medium is a safer method. The advantage of the method is that the disease-free plant material is available throughout the year. However, it is mainly suitable for medium-term conservation, as the cultures need regular maintenance, *i.e.* transfer to new nutrient medium and prolonged *in vitro* culture may add to the genetic instability of the material (Dulloo et al. 2010). In cryopreservation, the most laborious and therefore costly part is the initial phase – the preparation of the plant material and application of cryopreservation (Benson et al. 2011). However, after the material is cryopreserved, no other maintenance activities except ensuring the ultra-low temperature are needed (González-Benito et al. 2004; Bamberg et al. 2016). Thus, cryopreservation is the most secure and economically feasible method for long-term conservation of potato genetic resources. The material is, however, not readily available for research needs or breeders nor is it suitable for short- and medium-term preservation purposes (Benson et al. 2011). Only a small area of cells in the meristematic dome and leaf primordia survive cryopreservation (Ding et al. 2008; Kaczmarczyk, Rutten, *et al.* 2008; Wang *et al.* 2014a; Wang *et al.* 2014b) and therefore the regeneration into a sufficient amount of plants is time-consuming. Recently, cryopreservation applied for preservation of potato genetic resources has increased in plant gene banks (Kaczmarczyk *et al.* 2011; Niino & Arizaga 2015; Vollmer *et al.* 2016). This is possible due to continuous development and refinement of the cryopreservation protocols undertaken since the late 1970’s (Benson 2004), which has resulted in several effective cryopreservation methods (Reed 2008; Kaczmarczyk *et al.* 2011). However, the main challenge is still the variability in cryotolerance among different genotypes. For example, the average
regeneration rate for 1028 cryopreserved Solanum genotypes ranged from 34 to 59%, and for 70% of the accessions, the recovery rate exceeded 20% (Vollmer et al. 2016).

The key to minimizing the variability between accessions and extending the cryopreservation protocol for recalcitrant genotypes relies on understanding the fundamental biological processes related to cryotolerance (Funnekotter et al. 2017). Recently, several studies have specified the oxidative stress events during the cryopreservation process at the molecular level. For example, Zhang et al. (2015) revealed the responses of oxidative stress and the associated programmed cell death occurring during cryopreservation. Reduction of oxidative stress by application of antioxidant cinnamtannin B-1 or inhibition of apoptosis-like events by deactivating proteases increased the cell viability of embryogenic callus of Agapanthus praecox from 49 to 87 or 90%, respectively (Zhang et al. 2015). The study by Ren et al. (2015) elaborated the antioxidant status and the effect of application of exogenous antioxidants on cryopreservation success of Arabidopsis seedlings at the whole transcriptome level. Gross et al. (2017) defined the effect of various cryoprotectants (PVS2 and PVS3) on transcript abundance during the cryopreservation procedure for Arabidopsis shoot tip-derived seedlings.
1.2 Light as a source of environmental information

For optimal growth and development, all organisms perceive and process information about their biotic and abiotic environment. For plants, as sessile photosynthetic organisms, the information about light conditions is particularly important, as the light affects every aspect of plant growth and development (Fankhauser & Chory 1997; Fiorucci & Fankhauser 2017). Besides providing energy for photosynthesis, light intensity, direction, duration and spectral quality (composition of wavelengths) of light forms a source of information for regulating numerous plant physiological and metabolic processes (Gilmartin et al. 1990; Devlin et al. 2007; Huché-Thélier et al. 2016). For example, germination, de-etiolation, flowering, phototropism, stomatal opening, shade avoidance, organ development and elongation growth are all light-regulated processes (Fankhauser & Chory 1997). Compared to photosynthetically active radiation (PAR) (400–700 nm), which is also in the visible light range for the human eye, the wavelengths inducing photomorphogenetic responses range from near-UVB to far-red (~280 to ~750 nm) (De Wit et al. 2016). The composition of photomorphogenetic wavelengths – i.e. the light spectral quality – affects the quality and quantity of plants and is therefore intensively studied in relation to crop production, storage and protection (Ballaré & Casal 2000; Folta et al. 2005; Morrow 2008; Zoratti et al. 2015). For example, a controlled amount of light with a modified spectrum improves the nutritive quality and mitigates senescence and fruit ripening during postharvest storage (reviewed by D’Souza et al. 2015). Moreover, light spectrum affects the synthesis of bioactive compounds and antioxidants of many horticultural crops (reviewed by Hasan et al. 2017). The exposure to monochromatic wavelengths (e.g. blue, green or red) can even prevent microbial growth and induce systemic acquired resistance against pathogens in various plant species (D’Souza et al. 2015; Hasan et al. 2017).

Light conditions are sensed through specific photoreceptors detecting certain wavelengths (phytochromes, crytochromes, phototropins, ‘Zeitlupes’, UVR8). However, the following photosresponses are a result of a complex signal transduction network (Gilmartin et al. 1990; Casal & Yanovsky 2005; Vandenbussche et al. 2005; Devlin et al. 2007; Chaves et al. 2011; Galvã & Fankhauser 2015; OuYang et al. 2015; Wang & Wang 2015). Physiological responses to spectral changes can vary among different plant species, varieties, and even growth phases (Seabrook 1987; Benson et al. 1989; Goto 2003; Dutta Gupta & Jatothu 2013; Manivannan et al. 2015; Hasan et al. 2017).
1.2.1 Light in vitro

*In vitro* plants are generally not autonomous for photosynthesis but depend on a source of organic carbon, such as sucrose, added to the growth medium (Marks & Simpson 1999; George *et al.* 2008; Badr *et al.* 2011). For example, photomixotrophic *in vitro* potato plants (*S. tuberosum* cv Norland) have low photosynthetic capability, low CO₂ conductance and a distinct metabolite profile compared to photoautotrophic plants (Badr *et al.* 2011). Similar to photoautotrophic plants, light intensity, spectral quality and duration affect the photomorphogenetic development and quality of *in vitro* plants (Read 1988; George *et al.* 2008; Dutta Gupta & Jatothu 2013). Altered spectral composition affects the growth and morphogenesis, chlorophyll content, sucrose, starch, soluble sugar accumulation, as well as size and number of stomata in the leaves of *in vitro* plants (Dutta Gupta & Jatothu 2013; Liu *et al.* 2014). Moreover, monochromatic red or blue LEDs, separately or in combination, increase accumulation of phenolic antioxidants in several medicinal species (Liu *et al.* 2014; Manivannan *et al.* 2015; Pedroso *et al.* 2017). Therefore, the light quality greatly affects the physiological status and performance of *in vitro* cultures.

1.2.2 Light and cryopreservation

Light conditions play a significant role in plant germplasm cryopreservation. For several potato cultivars, recovery was higher when the plants originated from high light intensity conditions (Benson *et al.* 1989; Yoon *et al.* 2006). However, intense light before cryopreservation was not beneficial for all potato species/cultivars. For *Lomandra sonderi* (F. Muell) Ewart (Asparagaceae), an endemic plant of south-west Western Australia, low light conditions prior to cryopreservation resulted in a significant increase in recovery percentages compared to high light conditions (Funnekotter *et al.* 2016).

During the initial post-cryopreservation recovery, intense light has an adverse effect on survival of plant germplasm. The survival of *in vitro* shoot tips of *Garcinia mangostana* L. increased from 7%, without dark incubation to 25%, 30% or 50% survival after 3-, 5- or 7-day dark incubation, respectively, before transfer to light (22 µmol m⁻²s⁻¹) (Ibrahim & Normah 2013). Similarly, the recovery of embryos of *Zizania palustris* increased from 35% to 56% when recovered in darkness (Touchell & Walters 2000). Also, the post-cryopreservation viability of the freshwater diatom *Planothidium frequentissimum* and several Chlorophyte microalgae species depend
on light levels (Buhmann et al. 2013; Bui et al. 2013). For potato, post-
cryopreservation light levels affect recovery, but the requirements depend on
cultivar, recovery phase and cryopreservation protocol. Intense light was
detrimental to the survival of the potato cultivar Désirée during the first week after
thawing. However, increase in light intensity was important during the later
recovery phase to support morphological development (Benson et al. 1989). In
contrast, for the cultivar Golden Wonder, the highest recovery was obtained in high
post-freeze light when the ultra-rapid freezing protocol was used, and a low
survival was related to a controlled freezing followed by low light conditions
(Benson et al. 1989).

Too-intense light increases the production of reactive oxygen species (ROS),
which damage the photosynthetic apparatus (Müller et al. 2001; Domingues et al.
2012; Ahmad 2014). Post cryopreservation, the photosynthetic capacity can be
significantly reduced due to freezing-induced structural and functional damage in
chloroplasts. For example, protocorm-like bodies of several orchid species remain
green when incubated in darkness but undergo bleaching or browning when
exposed to light during initial post-cryopreservation recovery (Bratonia orchid,
Dendrobium nobile Lindl. (Bukhov et al. 2006; Mohanty et al. 2012). In general,
to avoid the light-related oxidative stress after cryopreservation, a period of full
darkness or diffuse light is applied from a few days to several weeks after
cryopreservation (Gonzalez-Arnao & Engelmann 2006; Reed 2008; Zhao et al.
2008).

These studies indicate that good recovery may not be possible in plants that
have been subjected to sub-optimal, pre-, and post-freeze light conditions.
Considering the pivotal role of light intensity on cryopreservation and the
significant effect of light spectral quality on plant development, morphogenesis and
physiological status, it is surprising that there is no information on the effect of
light spectral quality on cryopreservation success of plant germplasm. Until now,
the pre- and post-culture of cryopreserved material has been carried out under
fluorescent tubes, which are (at least this far) the most common light sources in in
vitro culture rooms. Light emitting diodes (LEDs) with their fully controllable
spectral emission, low heat emission, and energy consumption are superior light
sources for illumination in controlled growth conditions. Therefore, there is an
increasing trend to introduce LEDs into tissue culture chambers. However, a
change in the illumination type will cause a change in light spectral quality, which
may affect the in vitro plants and their cryopreservation success. On the other hand,
modification of light spectral quality may provide an additional tool to adjust the
light conditions both prior to and post-cryopreservation, which may significantly improve the cryopreservation success of plant germplasm.
2 The aim of the study

Despite the facts that light is one of the most important factors affecting growth and morphogenesis of plants, in general, and specific wavelengths drive specific responses during the different developmental stages, the effect of light spectral quality on cryopreservation success has not been studied before. The present thesis aimed to determine whether and how different light spectra applied before or after cryopreservation influence recovery and regeneration of potato shoot tips \textit{in vitro}. Through two original papers (I–II) and a manuscript (III), I searched for answers to the following questions:

1. Does pre-cryopreservation light quality affect cryopreservation success of potato shoot tips?
2. Does post-cryopreservation light quality affect survival and regeneration of potato shoot tips?
3. How do cryopreservation and different light conditions affect gene expression of recovering potato shoot tips at the whole transcriptome level?
3 Material and methods

The material and methods presented here are described only briefly. The full details can be found in the original papers (I–II) and in the manuscript (III).

3.1 Plant material

For cryopreservation experiments (I, II), in vitro plants of potato (Solanum tuberosum L.) cultivars Agrie Dzeltenie, Bintje, Maret, Anti and Désirée were used. The transcriptome profiling (III) was done for the cultivar Agrie Dzeltenie. The in vitro plants were obtained from the potato genebank of the Estonian Crop Research Institute (ECRI). The cultivar selection was based on their in vitro growth characteristics: cv Agrie Dzeltenie representing a fast-growing, cv Maret a medium-growing, and cv Anti a slow-growing cultivar. Cultivars Désirée and Bintje, which also belong to the medium-growing cultivars were included to allow comparison with the results of other cryopreservation studies dealing with potato cryopreservation. In the field, the growth characteristics are similar to in vitro conditions: Agri Dzeltenie and Mare are fast-growing early cultivars, cvs Désirée and Bintje medium-growing and Anti is a late cultivar with short and compact stems. The material was multiplied by placing nodal cuttings on MS medium with 2% sucrose, 6.4 g/l agarose at pH 5.7 without growth regulators and placed in 250-ml aerated plastic jars (Combiness). The cultures were kept in a growth room at 22°C and 16/8-h photoperiod under cool white fluorescent tubes (Philips TL-D 840) at 40 µmol m⁻² s⁻¹.

3.2 Light treatments

Six different light treatments were applied either before or after cryopreservation to dissect the effect of pre- and post-cryopreservation light quality. In study I, prior to cryopreservation, the nodal cuttings were exposed for 3.5 weeks to cool white fluorescent tubes (CW), hydrargyrum quartz iodide, i.e. warm white (HQI), white LEDs (W), blue LEDs (B), red LEDs (R) or a combination of 90% red and 10% blue LEDs (RB), while post-cryopreservation recovery was carried out under CW (Fig. 2). In study II, prior to cryopreservation, the nodal cuttings were exposed to CW (for 4 weeks), and the light treatments were applied during recovery. In study II, the light treatments were the same as in study I, except instead of white LEDs (W), a treatment of 90% red and 10% blue LEDs (of PAR) with 20% far-red (RBF)
was included (Fig. 3). In study III, before cryopreservation, the nodal cuttings were illuminated by CW for 4 weeks and the light treatments (16 hours of 40 µmol m⁻²s⁻¹ CW, RB, or total darkness (D)) were started after 48-h dark-incubation post rewarming (Fig. 3). In all three studies, the light intensity before cryopreservation was 40 µmol m⁻²s⁻¹. The post-cryopreservation recovery in study I was carried out by dark-incubation (one week) followed by exposure to low light conditions (20 µmol m⁻²s⁻¹ for one week) before transfer to the standard conditions (40 µmol m⁻²s⁻¹ CW). In study II, the shoot tips were exposed to low light conditions (20 µmol m⁻²s⁻¹) for 2 days before the light intensity was increased to 40 µmol m⁻²s⁻¹.

The reported light quality, as well as intensity, was measured by spectroradiometer (OceanOptics USB2000+RAD) at the level of explants outside the vessel. The culture vessel material (Combiness plastic jars and Petri plates) did not affect the light quality. Inside the vessel, the light intensity was approximately 2 µmol m⁻²s⁻¹ lower than outside the vessel.

Fig. 2. The light quality treatments applied either during pre- or post-cryopreservation culture of potato shoot tips in vitro. CW – cool white fluorescent tubes, HQI – warm white, W – white LEDs, B – blue LEDs, R – red LEDs, RB – a combination of red and blue LEDs (90% red, 10% blue), RBF – the same as RB with 20% far-red.
3.3 Morphological measurements

In study I, 10–15 plants were measured per five cultivars divided into six light quality treatments after 3.5-weeks exposure to each light treatment. The measured characteristics were the shoot and root lengths, as well as fresh weight (FW) and the number of axes.
3.4 Cryopreservation

In all three studies, cryopreservation was done by the DMSO-droplet method originally developed by Schäfer-Menuhr et al. (1997) and further improved by Kaczmarczyk et al. (2008). On the first day, 1–3 mm shoot tips were excised under stereomicroscope and incubated overnight on filter paper (ø 4.5 cm) moistened with 2 ml MS solution (Murashige & Skoog 1962) including 3% sucrose in 5.5-cm-diameter Petri dishes (Fig. 4). The next day, the shoot tips were cryoprotected by incubating in 10% DMSO in liquid MS with 3% sucrose for 2 h. After that, the shoot tips were transferred to sterile foil strips carrying 2.5 µl droplets of cryoprotectant, cooled by transferring the foil strips into cryotubes filled with liquid nitrogen (LN) and stored in an LN container for 1 h. The rewarming was carried out by transferring the foils into 30 (I, II) or 50 (III) ml of MS solution at room temperature (RT). For recovery, the shoot tips were placed on regeneration medium (MS medium with 0.5 mg/l zeatin riboside, 0.2 mg/l GA₃ and 0.5 mg/l IAA, 30 g/l sucrose). Non-cryopreserved controls were treated like the cryopreserved ones without the cooling step. The survival (viable shoot tips without shoot formation) and regeneration (shoot tips with new green shoots) were evaluated 2, 4 and 6 weeks after cryopreservation (Fig. 4). In all three studies, only apical shoot tips were used for cryopreservation.
3.5 RNA extraction and sequencing (III)

For transcriptome profiling, 150 shoot tips from each treatment (non-cryopreserved control shoot tips exposed to CW, cryopreserved shoot tips exposed to CW, RB, or D) were collected and placed into RNAlater stabilization reagent (Qiagen) under ambient light conditions. The cultivar Agrie Dzeltenie was selected for the transcriptome study because it had the highest regeneration percentage among all cultivars in the post-cryopreservation treatment with RB. The shoot tips were transferred to -80°C until RNA extraction. RNA was extracted from 10 shoot tips originating from the same culture vessel and replicated three times for each treatment, therefore forming three biological replicates per treatment. The total RNA was isolated by RNeasy Plant Mini Kit (Qiagen) and purified by RNase-free DNase Set (Qiagen). The quality and quantity of total RNA were assessed using Agilent Bioanalyzer 2100 (Agilent Technologies), and only high-quality samples (RIN >7.4) were used for library preparation using Illumina’s TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA). The 12 paired-end libraries were sequenced by Illumina HiSeq2500 by 100 cycle mode in the Finnish Functional Genomics Centre of the Turku Centre for Biotechnology.
3.6 Statistical and bioinformatics analyses

3.6.1 Growth characteristics (I)

The growth characteristics of *in vitro* plants (I) were analysed by analysis of variance (ANOVA) followed by multiple comparisons of means by Tukey contrasts by the Rcmdr package (Fox 2005). Samples that did not fulfil assumptions for ANOVA were analysed by the Kruskal-Wallis test followed by Dunn's test (Dunn 1964) using the dunn.test package (Dinno 2016). *P*-values of pairwise comparisons were corrected by Holm's correction (Holm 1979). For visualization, the ggplot2 package was used (Wickham 2009). The analyses were performed with statistical programming language R (R Core Team 2015).

3.6.2 Cryopreservation (I, II)

The counts of survived, regenerating and dead shoot tips in each light quality treatment were analysed in contingency tables by a Chi-squared test of independence followed by pair-wise comparisons by a Fisher exact test with Holm's *p*-value correction (Holm 1979) performed by the R package fmsb (Nakazawa 2015). For visualization, the residual-based mosaic plots generated by the vcd package were used (Meyer *et al.* 2013). The analyses were performed in the R statistical environment (R Core Team 2015).

3.6.3 Bioinformatics analysis

The quality of reads was checked by FastQC (Andrews 2010) and aligned to the *S. tuberosum* reference genome (PGSC v4.03) (The Potato Genome Sequencing Consortium 2011; Sharma *et al.* 2013) by TopHat and bowtie1 (Langmead *et al.* 2009; Trapnell *et al.* 2009). The reads per gene were counted based on the TopHat output using featureCounts (Liao *et al.* 2014). For differentially expressed gene (DEG) detection between different treatments (III, Fig. 1), DESeq2 was used (Anders & Huber 2010; Love *et al.* 2014). A false discovery rate (FDR) was estimated based on Benjamini Hochberg correction (Benjamini & Hochberg 1995), and only genes with a FDR of <0.05 were considered significant. The similarities and distances between samples were analysed and visualized by principal component analysis (PCA) and a heatmap (Yao *et al.* 2012; Love *et al.* 2014). Gene Ontology (GO) enrichment analysis of DEGs in each treatment comparison was
performed using the PlantRegMap (PlantTFDB v4.0) GO enrichment tool (Jin et al. 2016), and it was visualized by R package pheatmap (Kolde 2015) and BiNGO plug-in for Cytoscape (Maere et al. 2005). GO terms with corrected $p$-values <0.05 were considered significantly enriched.
4 Results

The results presented here are described only briefly. The full details can be found in the original papers (I–II) and the manuscript (III).

4.1 The effect of pre-cryopreservation light quality (I)

4.1.1 Growth characteristics (I)

All light quality treatments produced visibly healthy and vigorously growing *in vitro* plants. However, the light quality significantly affected the growth and morphology of all cultivars tested. The most prominent response in growth characteristics was shoot elongation. R and RB promoted shoot elongation while the shortest plants were recorded in broad-spectra treatments, specifically under W (I, Fig. 2 and Fig. 3). R affected primarily the elongation growth, as for most of the cultivars, the number of axes was not affected by the different light treatments, although the *cv* Maret had a significantly lower number of axes in R. Root elongation was induced only in the *cv* Désirée, which had the longest roots in HQI and shorter in R and RB.

Among the other measured growth characteristics, light spectral quality significantly affected biomass accumulation. The shoot and root biomasses were higher in plants treated with broad wavelengths compared to narrow wavelength treatments. However, different cultivars responded slightly differently, as significant changes were observed only for *cvs* Agrie Dzeltenie, Désirée and Anti, and not for *cvs* Bintje and Maret. Specifically, the highest shoot fresh weights were recorded in W for *cvs* Anti and Désirée, and in HQI for *cv* Agrie Dzeltenie. In addition, the highest root fresh weight was recorded under W for *cvs* Agrie Dzeltenie and Anti, while for *cv* Désirée, in HQI. The lowest shoot biomass was found in B for *cv* Anti, and in RB for *cvs* Désirée and Agrie Dzeltenie. The lowest root biomass accumulation occurred under B and RB for *cv* Anti, RB for *cv* Désirée, and RB and R for *cv* Agrie Dzeltenie.

4.1.2 Cryopreservation (I)

Some of the pre-cryopreservation light treatments significantly affected the post-cryopreservation recovery, but the specific responses varied during the 6-week
follow-up period between different cultivars. Under B, high early recovery (2 weeks post-cryopreservation) was observed for all cultivars tested, whereas under R or HQI, depending on cultivar, the lowest survival percentages were obtained 2–4 weeks after thawing. Specifically, 2 weeks post-cryopreservation, the highest survival was in B-grown shoot tips with 66% survival compared to 48% in CW and 26% in R for cv Agrie Dzeltenie. Similarly, for cv Anti, the highest survival of 31% was observed in B-grown shoot tips 2 weeks post-cryopreservation, compared to 22% in CW and 4% in R. The same trend was observed for cv Désirée with the highest survival of 48% in B, 9% in CW and only 2% in HQI (I, Fig. 4). For cv Bintje, the recovery was 20% in CW, while in B and RB it was 41% and 44%, respectively. For cv Maret, the regeneration was 40% in CW, and in B and R 62% and 63%, respectively. However, the differences in survival percentages decreased during the 6-week follow-up period. This was caused by a decline in recovering shoot tips starting either 2, 4 or 6 weeks post-cryopreservation, depending on cultivar (I, Fig. 4). The green recovering shoot tips turned brown, and some exhibited signs of contamination (I, Supplementary material). The first regenerating plants were recorded 2 weeks post-thawing for cv Désirée and 4 weeks post-thawing for other cultivars. Cv Désirée was also the only cultivar, which showed different regeneration percentages in plants originating from different light treatments. Specifically, 6 weeks post-cryopreservation, the highest regeneration for cv Désirée was in RB with 19% regenerating shoot tips while in CW the regeneration percentage was only 5%. For cv Agrie Dzeltenie, the regeneration percentages from different light treatments ranged from 10% to 21% and for cv Bintje from 21% to 33%. For cvs Anti and Bintje, the regeneration percentages remained lower and varied from nil to 3% and 5%, respectively (I, Fig. 4).

4.2 The effect of post-cryopreservation light spectral quality (II, III)

4.2.1 Cryopreservation (II)

Post-cryopreservation light quality significantly affected regeneration of all cultivars tested, while for most cultivars, light quality did not affect the survival percentages. The highest regeneration percentages were obtained for all cultivars under RB, while R resulted in low regeneration for most cultivars. In RB, the regeneration percentages increased in comparison to CW from 26% to 53% for cv
Agrie Dzeltenie, 25% to 44% for cv Maret, 8% to 26% for cv Bintje, 0% to 17% for cv Anti and 18% to 36% for cv Désirée (II, Fig. 2). At the same time, the regeneration percentages in R were 3% and 11% for cvs Désirée and Maret, respectively, while no regeneration was found in R for cv Anti. In addition to R, low regeneration was observed in HQI for cv Agrie Dzeltenie (12%) and in RBF for cvs Maret and Bintje (6% and 1%, respectively). The monochromatic B had a genotype-specific effect. For cv Agrie Dzeltenie, the regeneration in B (47%) reached the same level as in RB (52%), but for cv Bintje, the shoot regeneration was significantly lower in B (2% regenerating). Simultaneously, B did not affect the regeneration of cvs Maret, Désirée and Anti.

In general, the survival percentages did not differ between the light quality treatments. The only differences found were in CW for cv Agrie Dzeltenie and HQI for cv Bintje, where the survival remained lower (58% and 26%, respectively) in comparison to the average of other light treatments (II, Table 2).

4.2.2 Transcriptome (III)

**Light effect: CW+ and RB+ compared to D+**

Based on the transcriptome analysis, post-cryopreservation light conditions significantly affected the gene expression of recovering shoot tips, as 3,284 genes were significantly up- or down-regulated in CW+ and RB+ when compared to D+ (III, Fig. 3B). In CW+, the number of both up- and down-regulated genes (1,923 and 976, respectively) was double compared to RB+ (1,024 up- and 462 down-regulated). Moreover, most of the RB+ -up-regulated differentially expressed genes (DEGs) were the same as in CW+ (867), and only 157 were specific to RB+. At the same time, the number of CW+ -specific DEGs was 1,056. Also, among the down-regulated genes, most of DEGs were specific to CW+ (742), while 234 were common to CW+ and RB+, and 228 to D+ (III, Fig. 3B).

Gene ontology (GO) analysis revealed that the main up-regulated biological processes (BP) in both light treatments were related to photosynthesis, response to light stimulus and response to high light intensity. Most of these responses were more pronounced in CW+ than in RB+ (III, Fig. 6A, B and C). The top enriched GO terms common to both light treatments were ‘photosynthesis’, ‘photosynthesis, light reaction’, ‘generation of precursor metabolites and energy’, ‘photosynthetic electron transport chain’ and ‘electron transport chain’.
Moreover, in CW+, several repair and assembly processes (‘photosystem II assembly’, ‘protein complex assembly’, ‘protein complex biogenesis’, ‘cellular component assembly’, ‘photosystem II oxygen-evolving complex assembly’) as well as defence responses (‘defence response to bacterium’, ‘response to bacterium’, ‘response to stress’, ‘response to redox state’) were activated. In RB+, secondary metabolite processes (‘secondary metabolic process’, ‘phenylpropanoid metabolic process’, ‘phenylpropanoid catabolic process’) were activated (III, Fig. 6D, E and F). Among the other responses, ‘response to karrikin’ was activated in both CW+ and RB+ in comparison to D+ (Fig. 6G). No significant GO term enrichment was found for down-regulated DEGs in light compared to darkness.

The differences between CW+ and RB+

The shoot tips recovering in RB+ had 495 DEGs when compared to CW+. Specifically, in RB+, 158 genes were up- and 340 down-regulated. GO analysis indicated that in RB+, photosynthesis and several stress and defence responses were down-regulated (III, Fig. 7), while the up-regulated DEGs referred to activation of shoot system development and organ morphogenesis (III, Supplementary Fig. 2). The top morphogenesis-related GO terms were ‘regionalization’, ‘regulation of meristem growth’, ‘pattern specification process’, ‘anatomical structure arrangement’ and ‘determination of dorsal/ventral symmetry’ (III, Supplementary Fig. 2). The top enriched GO terms related to down-regulated DEGs were ‘photosynthesis’, ‘response to abiotic stimulus’, ‘response to temperature stimulus’, ‘generation of precursor metabolites and energy’, ‘response to stimulus’ and ‘response to stress’ (III, Fig. 7).

Cryopreservation effect: CW+, RB+ and D+ compared to CW-

Cryopreservation had an extensive effect on the transcriptome profile of potato shoot tips. In total, 12,017 DEGs were significantly up- or down-regulated in the cryopreserved shoot tips (CW+, RB+ and D+) in comparison to non-cryopreserved ones (CW-) (III, Fig. 3A). Half of the up-regulated DEGs were common to all cryopreserved samples (3,040), 589 were specific to CW+, 314 to RB+ and 861 to D+. Among down-regulated DEGs, 2,738 were common to all cryopreserved samples while 675 were specific to CW+, 276 to RB+ and 1,081 to D+ (III, Fig. 3A).
GO enrichment analysis indicated that cryopreservation activated numerous stress and defence responses (III, Fig. 4), while the main down-regulated biological processes (BP) were related to morphogenetic development (III, Supplementary Fig. 1). The top enriched GO terms among up-regulated DEGs were ‘response to biotic stimulus’, ‘response to external biotic stimulus’, ‘response to other organism’, ‘multi-organism process’ and ‘defence response’ (III, Fig. 4). The top GO terms related to down-regulated DEGs were ‘shoot system development’, ‘reproductive shoot system development’, ‘anatomical structure morphogenesis’, ‘post-embryogenic plant morphogenesis’ and ‘regulation of organ morphogenesis’ (III, Supplementary Fig. 1)
5  Discussion

5.1  Growth characteristics (I)

The growth characteristics of five potato cultivars under different light quality treatments were studied to define the association between photomorphogenetic responses and the subsequent cryopreservation success. Most of the growth characteristics are in line with the previous studies carried out on \emph{in vitro} plants of potato in different light wavelengths. The 52\% increase in average shoot length of five potato cultivars in red light (I) is comparable to the average of 58\% increase of the three most common commercial potato cultivars in Brazil (Asterix, Catucha, Macaca) and 59\% of four North American cultivars (Norland, Superior, Kennebec, Denali) after 4 weeks in red light compared to fluorescent tubes (Wilson \emph{et al.} 1993; Rocha \emph{et al.} 2015). The effect of red light on \emph{in vitro} shoot elongation is well known not only for potato (Wilson \emph{et al.} 1993; Aksenova \emph{et al.} 1994; Seabrook 2005; Rocha \emph{et al.} 2015) but also for a broad range of species (Goto 2003; Kim \emph{et al.} 2004).

Blue light reduces cell expansion and thus inhibits stem elongation and leaf growth (Cosgrove & Green 1981). This has been proven for the Brazilian, as well as North American cultivars (described above), which produced the shortest \emph{in vitro} shoots in blue light (Wilson \emph{et al.} 1993; Rocha \emph{et al.} 2015). However, for some cultivars (Asterix and Kennebec), the shoot length in blue light was comparable to that seen under fluorescent lamps or VitaLite fluorescent tubes (Wilson \emph{et al.} 1993; Rocha \emph{et al.} 2015). Also, Aksenova \emph{et al.} (1994) reported that blue light suppressed \emph{in vitro} stem growth of the cultivar Miranda in comparison to red light. In the present study, for all cultivars, the shortest plants were observed under white LEDs, while blue light-treated shoots did not differ from those of broad-spectra light treatments. This may be explained by the different light intensities used by Rocha \emph{et al.} 2015 (20 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)) and Wilson \emph{et al.} 1993 (100 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)) compared to the 40 \( \mu \text{mol m}^{-2}\text{s}^{-1} \) and different cultivars used in the present study. Moreover, Aksenova \emph{et al.} (1994) claimed that the blue-grown plantlets were short, thick and had large, well-developed leaves. In the present study, the blue-grown shoots appeared thin and tiny. The difference is likely the result of different light intensity, as Aksenova \emph{et al.} (1994) had four times higher PPF (160 \( \mu \text{mol m}^{-2}\text{s}^{-1} \)).
5.2 Cryopreservation (I, II)

The present study revealed that both pre- and post-cryopreservation light spectral quality significantly affect the cryopreservation result of potato shoot tips in vitro. Monochromatic wavelengths alone or in combination (R, B, or RB) had the most prominent effect on cryopreservation success, but the beneficial light qualities differed between pre- and post-cryopreservation culture. Specifically, blue light-grown shoot tips had high recovery two to four weeks after thawing, while post-cryopreservation recovery under RB doubled the regeneration percentages of all cultivars. At the same time, for most cultivars, monochromatic red LEDs were adverse both before and after cryopreservation.

For a genebank, the success of a shoot tip/meristem cryopreservation protocol is defined by the percentage of plant material able to resume its growth, e.g. to form new shoots (Benson & Harding 2012; FAO 2014; Vollmer et al. 2016). By these values, some of the post-cryopreservation light quality treatments directly affected the cryopreservation success of potato shoot tips in the present study. In RB, the average regeneration percentage of all cultivars increased by 56%, while in monochromatic R the regeneration reduced by 40% in comparison to cool white fluorescent tubes CW (II).

There are no previous studies on the effect of light spectral quality in relation to cryopreservation of plant germplasm. Therefore, the responses to various light spectra can only be compared to the studies done on in vitro growth characteristics without cryopreservation. However, the evaluation is not straightforward because the reaction depends not only on the species and cultivar, but also on the developmental stage, origin of explant material, photosynthetic photon flux (PPF) and growth medium (Seabrook 1987; Benson et al. 1989; Aksenova et al. 1994; Schuerger et al. 1997; Nhut et al. 2003; Kim et al. 2004; Kurilčík et al. 2008; Dutta Gupta & Jatothu 2013; Manivannan et al. 2015).

Of the in vitro growth characteristics, in study I the highest shoot elongation occurred under R LEDs followed by RB LEDs. However, the exposure to R during post-cryopreservation recovery did not have a similar response. RB promoted the shoot formation the most, while for the majority of the cultivars, shoot formation was inhibited under R. The unfavourable effect of R light was observed even among non-cryopreserved controls (II). The survival and regeneration of control shoot tips were in general very high, but in R, the survival was 85% with 80% regeneration for cv Agrie Dzeltenie (II, Table 2). Therefore, in the present study, the light treatment that promoted the in vitro shoot elongation from nodal cuttings the most
(R) was not suitable for induction of shoot organogenesis of recovering meristems post-cryopreservation.

For successful cryopreservation, the source plants need to be in optimal physiological state (Engelmann 2004; Reed 2008). In the present study, all tested light spectral qualities produced visibly healthy in vitro plants, but the growth and morphology were significantly altered. The visually strongest and healthiest plants were grown in the wide-spectra treatments (CW, HQI, W), while the red-treated shoots were elongated, and blue-treated shoots remained thin and tiny. However, from the tested light quality treatments, blue-grown shoot tips resulted in high initial survival for all cultivars, while for most cultivars the red-grown shoot tips had low survival. Post-cryopreservation survival is a pre-requisite for regeneration, and therefore it may significantly affect the overall cryopreservation success, measured as the formation of new shoots.

In blue light, the cell wall expansion, elongation growth, as well as gibberellin and auxin synthesis and sensitivity are suppressed (Cosgrove & Green 1981; Wilson et al. 1993; Folta et al. 2003). For potato, the cells were the shortest in blue light and the longest in red light (Wilson et al. 1993). Therefore, the higher early survival may relate to the reduced elongation growth and smaller cell size caused by blue light. For cryopreservation, small cell size is beneficial due to small vacuoles with lower water content and high nucleo-cytoplasmic ratio (Engelmann 2004; Martinez-Montero & Harding 2015). However, in the present study, the cell morphology was not studied; therefore, this could be a subject of further study.

Light spectrum not only affects morphological characteristics but also significantly influences the accumulation of both primary and secondary metabolites as well as antioxidants (e.g. soluble sugars, starch, vitamin C, soluble protein and polyphenol composition) during in vitro culture (Hasan et al. 2017). For several medicinal plants, the modified light conditions affect the production of bioactive compounds, which increases their pharmacological value. For example, for critically endangered medicinal plant Swertia chirate, illumination by monochromatic blue LEDs resulted in the highest total phenolic, total flavonoid, and total flavonol content, as well as total antioxidant capacity. At the same time, in red light, these values were the lowest (Dutta Gupta & Karmakar 2017). For in vitro cultures of Plectranthus amboinicus (Lour.) Spreng, the highest content of the antioxidant compound carvacol was recorded in blue light, but in comparison to fluorescent tubes, the carvacol content was significantly higher also in monochromatic red and in the combination of RB (1:1). Interestingly, in other RB combinations (2.5B:1R and 1B:2.5R), the carvacol content was at the same level
as under fluorescent tubes (Silva et al. 2017). Therefore, the RB ratio significantly affects the metabolite as well as antioxidant accumulation in *in vitro* plants. In the present study, the tested LEDs were monochromatic R, B and a combination of RB with 9:1 RB ratio. The optimisation of RB ratio during both pre- and post-cryopreservation culture could significantly improve the cryopreservation efficiency of potato shoot tips *in vitro*.

In addition to metabolite and antioxidant content, light spectral quality also affects the reactive oxygen species (ROS) generation and the associated antioxidative system of *in vitro* plants (Dutta Gupta & Sahoo 2015; Dutta Gupta & Agarwal 2017). The *in vitro* plant recalcitrance is closely related to the balance between antioxidant status and oxidative stress caused by ROS (Benson 2000; Dutta Gupta & Datta 2003; Blazquez et al. 2009). However, ROS play a dual role in plant biology, as ROS are required for many important signalling reactions to abiotic stress responses and for progression of cellular proliferation and differentiation, while the over-production of ROS is detrimental, leading to lethal events (Mittler 2002; Mittler 2017).

In the study by Dutta Gupta and Sahoo (2015), irradiation by R, B and RB (1:1) significantly affected the *in vitro* shoot formation, as well as hydrogen peroxide and lipid peroxidation level (MDA content) and antioxidant enzyme activity (SOD, CAT, POX, APX, GR) of *Curculigo orchioides* Gaertn. In the monochromatic blue light, high shoot regeneration was accompanied by an increase of superoxide dismutase (SOD) activity, while in red light, the organogenesis was inhibited by the high level of hydrogen peroxide and lipid peroxidation in terms of MDA content. In R, the level of hydrogen peroxide and MDA level was more than double compared to other light treatments (Dutta Gupta & Sahoo 2015). In *in vitro* shoots of the medicinal plant *Rehmannia glutinosa*, the highest total phenol and flavonoid levels were obtained under monochromatic blue and red LEDs, respectively, which significantly correlated with the total antioxidant capacity of the leaf extracts. In addition, the highest antioxidant enzyme activity (SOD, GPX, APX, CAT and PAL) was obtained in blue light, followed by red LEDs in comparison to cool white fluorescent tubes (Manivannan et al. 2015). Therefore, the light spectral quality significantly modulates the cellular redox balance and significantly affects the *in vitro* shoot formation potential (Dutta Gupta & Agarwal 2017).

The cryopreservation procedure causes extreme stress, accompanied by the formation of ROS (e.g. superoxide radical $\text{O}_2^-$, hydrogen peroxide $\text{H}_2\text{O}_2$, singlet oxygen $^1\text{O}_2$), which may lead to excessive oxidative stress and thereby compromise the post-cryopreservation recovery (Chen et al. 2015; Zhang et al. 2015;
Funnekotter et al. 2017). As reported on cryopreservation of Ribes nigrum (Harding et al. 2009), Agapanthus praecox subsp. orientalis (Chen et al. 2016), and Arabidopsis thaliana seedlings (Ren et al. 2015), the enhancement of plant cellular antioxidant status may significantly increase the plant’s cryotolerance. As light quality significantly contributes to the cellular redox balance, it may be an additional parameter to optimize in cryopreservation protocols.

Despite the fact that blue-grown shoot tips showed high initial post-cryopreservation survival, the survival percentages declined depending on both cultivar and treatment at 4 or 6 weeks post-cryopreservation, and at the final evaluation of the experiment, no differences in regeneration percentages were found for most cultivars (I). In study II, no decline in survival, nor in regeneration percentages were observed during the 6-week follow-up period. The comparison of survival and regeneration of the control light treatment (CW) in both studies showed that some cultivars had lower survival and regeneration in study I in comparison to study II. The cultivar Anti had exactly the same survival and regeneration percentages in both studies in CW. Cultivars Maret and Agrie Dzeltenie had higher survival in study II (73% and 56%, respectively) compared to study I (35% and 42%, respectively), but the regeneration percentages in CW were similar in both studies. Cultivars Bintje and Désirée had higher survival in study II (73% and 56%, respectively) compared to study I (35% and 42%, respectively), but the regeneration percentages in CW were similar in both studies. Cultivars Bintje and Désirée had more differences – for both cultivars in study II, the survival was over 40%, whereas in study I, the final survival remained low (5%) and nearly zero regeneration in CW for Bintje and 5% for Désirée. From the studied cultivars, only Désirée has been cryopreserved by the DMSO-droplet method previously. The results vary similarly from 65% survival and 5% shoot formation (Harding et al. 1991), 41% survival and 25% regeneration (Schäfer-Menuhr et al. 1997), to 8% survival and 19% regeneration (Kaczmarczyk et al. 2008).

It is well known that sub-optimal physiological status may adversely affect cryopreservation success even if the cryopreservation protocol has been fully optimised. In the present study, several factors may have affected the performance of plants in study I. One such critical factor is the in vitro age. The younger in vitro cultures are known to have higher survival and regeneration percentages (Keller & Dreiling 2003; Harding et al. 2009). Harding et al. (2009) found significant differences in survival and regeneration percentages between cultures, which were initiated either 6–8 weeks or 3 years before the cryopreservation experiment (Harding et al. 1991; Harding et al. 2009). In the present study, the in vitro age between study I and II was approximately 3 years, study II being performed before study I, which may explain the differences in survival and regeneration percentages.
Therefore, older *in vitro* cultures may need regeneration/rejuvenation procedures, such as microtuber rejuvenation, to increase cryopreservation efficiency (Harding *et al.* 2009; Benson *et al.* 2011; Bamberg *et al.* 2016).

Another factor which may have reduced the survival and regeneration percentages (in study I) is the presence of latent endophytic bacteria, which may reside in the tissues without causing any sign of contamination under propitious growth conditions, but can become pathogenic under extreme stress (Podolich *et al.* 2009; Podolich *et al.* 2015). In long-term *in vitro* culture, covert endophytic bacteria may cause a decline in *in vitro* shoot and root growth (Thomas 2004) and increase the endophyte breakout frequency (Keller *et al.* 2011; Senula & Keller 2011; Edesi *et al.* 2017; Jenderek & Reed 2017). In study I, to avoid possible *in vitro* age and cryopreservation stress-related endophyte breakout, plant preservative mixture (PPM), a broad-spectrum biocide, was added to both pre- as well as post-cryopreservation growth media. Regardless, the first signs of endophyte breakout appeared 4 or 6 weeks after thawing. The signs of contamination were present only in some cultivars/treatments without a clear pattern, and some shoot tips lost their viability and turned brown without any sign of microorganism breakout (I, Supplementary material). Therefore, these results highlight the importance of *in vitro* plant quality as one of the most critical factors affecting post-cryopreservation recovery (Reed *et al.* 2004).

**5.3 Transcriptome characteristics (III)**

The transcriptome analysis of the non-cryopreserved and recovering shoot tips exposed to different light conditions (CW, RB, and D) was performed to explore why the shoot tip recovery was increased in RB when compared to the cool white fluorescent tubes (CW) (II), and how the presence of light affects the transcriptome profile of the recovering shoot tips.

The analysis revealed that cryopreservation induced expression of a myriad of stress and defence-responsive genes, which were more enhanced in CW+ than in RB+ and D+. Interestingly, the number of genes specifically activated in CW+, which is the commonly used illumination source during post-cryopreservation recovery, was much higher than in RB+ (1056 vs 157) when compared to D+ (III, Fig. 3B). In CW+, numerous stress and defence responses to both biotic and abiotic stimuli were abundant in comparison to shoot tips recovering in RB+. Photosynthesis and light stimulus-responsive genes were activated in both CW+
and RB+, whereas several morphogenesis-responsive genes were activated only in RB+ but not in CW+ (III, Fig. 6, Fig. 7 and Supplementary Fig. 2).

The light intensity in both light treatments was the same (40 µmol m⁻²s⁻¹) with the only difference being the spectral distribution. CW had a higher proportion of blue and less red, while 44% of the spectrum composed of green and yellow wavelengths (III, Supplementary Table 1). It has been shown that the yellow parts of the photosynthetically active radiation (PAR) may be harmful and cause damage to photosystem II (Takahashi et al. 2010). Alternatively, although the light treatments were carried out in a climate-controlled growth chamber, it is possible that the fluorescent tubes have emitted heat and caused a local increase in temperature, which may explain the photoinhibition and photosystem repair processes obvious in CW+.

The present study also enabled following the general transcriptional changes related to cryopreservation of clonally propagated in vitro shoot tips of potato. The analysis revealed the extent of the effect of cryopreservation on transcript abundance, as 64–73% of up- and 59–70% of down-regulated genes in each treatment were affected by cryopreservation, independent of light treatment (III, Fig. 3A). In the study by Ren et al. (2015), cryopreservation also resulted in a high number of DEGs in the recovery phase in comparison to non-cryopreserved seedlings. Therefore, both of these studies (II, Ren et al. 2015) illustrate the complex and extensive effect induced on the plant by cryopreservation.

The majority of up-regulated DEGs in all cryopreserved samples were related to stress, defence responses to biotic stimuli, and oxidative stress (III, Fig. 4 and Fig. 5). This may be related to the overall activation of quantitative defence responses caused by cryoinjury. However, the earlier transcriptome studies on Arabidopsis seedlings did not highlight activation of response to biotic stimulus (Ren et al. 2015; Gross et al. 2017). Therefore, the defence responses could be related to the preservation of clonally propagated tuber crops, usage of different cryopreservation protocols, or the presence of latent endophytic organisms. The breakout of endophytes occurring in study I was already discussed above. Therefore, it is possible that the biotic responses seen in gene expression were caused due to activation of hidden endophytes by the severe stress conditions of cryopreservation. However, the presence of endophytic organisms did not appear during study III. Therefore, it would be interesting to determine whether the activation of biotic defence-responsive genes was of a quantitative nature related to the overall cryopreservation stress or due to the presence of hidden endophytes. In addition, as the light spectrum has a general effect on the redox status of in vitro plants, a more-
focused study on the effect of light quality on the gene expression of recovering shoot tips and their antioxidant status might be of interest.
6 Conclusions and future prospects

The present results revealed the significant role of light spectral quality in cryopreservation success of potato germplasm. Specifically, five potato cultivars were cultivated in vitro under six different light qualities applied either before or after cryopreservation. Blue light-grown shoot tips had high initial recovery after thawing, while post-cryopreservation exposure to a combination of red and blue LEDs (90% red 10% blue, RB) doubled the regeneration percentages of all cultivars. At the same time, for most cultivars, monochromatic red LEDs had adverse effects both before and after cryopreservation culture. The present study also indicated that all light qualities produced healthy in vitro plants, but the morphology was altered. In general, monochromatic R and RB promoted elongation growth while biomass accumulation was higher in broad-spectra illumination. In the present thesis, light quality treatments were applied either before or after cryopreservation. The combination of beneficial pre- and post-cryopreservation light treatments (e.g., blue prior and RB post cryopreservation) could further improve the cryopreservation efficiency. However, thorough studies on a variety of different genotypes and combinations of red and blue are needed to find the optimal light quality requirements regarding cryopreservation. Moreover, in addition to the DMSO-droplet method used in the present thesis, the effect of different light quality treatments should be tested with other commonly used cryopreservation methods, e.g., droplet vitrification.

To understand the processes behind the positive effect of RB LEDs on shoot tip regeneration, a whole transcriptome analysis of recovering shoot tips of the cultivar Agrie Dzeltenie was performed. The transcriptome analysis revealed that most of the activated responses in recovering shoot tips exposed to RB were related to the activation of organ morphogenesis processes while at the same time several stress and defence-responsive genes were down-regulated when compared to cool white fluorescent illumination. These results suggest that the optimisation of light spectral quality during the recovery phase may reduce light-related stress levels while simultaneously supporting morphogenesis towards new shoot formation. However, the results are based on bioinformatics study of transcriptome data and need to be confirmed by quantitative real-time PCR, before final conclusions can be made.

The transcriptome analysis also enabled, for the first time, following the general transcriptional changes related to cryopreservation of potato – the most important clonally propagated food crop in the world. The results emphasize the
complex and extensive effect of cryopreservation on gene expression and accentuate the activation of various defence responses to biotic stimuli and oxidative stress. In the future, the reason for activation of defence responses to biotic stimuli could be uncovered by microbiological analyses of the cryopreserved samples. These findings may help to further understand the critical steps needed for successful cryopreservation of clonal crops and therefore improve the cryopreservation efficiency of plant germplasm in the future.

The present thesis reveals that light spectral quality is an additional non-cryogenic factor, which can significantly increase the cryopreservation efficiency of plant germplasm. However, the reasons behind the observed responses need to be further studied to understand the processes behind successful cryopreservation. For example, the effect of light quality on shoot tip ultrastructure, metabolite composition and redox balance could shed light on the observed effects in the present thesis.
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