An Investigation into Ploidy and Polyploidy in the *Sphagnum* Genus, a Screening of Micropropagted material and wild material:

A Study

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Declaration of originality

This is to certify that the work is entirely my own and not of any other person, unless explicitly acknowledged (including citation of published and unpublished sources). The work has not previously been submitted in any form to the Manchester Metropolitan University or to any other institution for assessment or for any other purpose.

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Abstract

Ploidy and polyploidization plays an essential role in the evolutionary diversification of plants. An evolutionary trait that not only continues the plants habitats and functions. but can initiate the rebuilding of habitats, biodiversity and sequester atmospheric carbon. The *Sphagnum* genus is no exception to this and perhaps one of most understood when it comes to its genetic advantages. Investigations of *Sphagnaceae* ploidy and genome sizes using flow cytometry are predominantly featured in studies analysing a wide range of bryophytes. Studies analysing *Sphagnaceae* specifically are few and protocol of extraction and analysis tend to be nondisclosed. The potential of polyploidy and its presence in non-hybridized *Sphagnum* has both evolutionary and climatic benefits, although it's theorised that natural polyploidy is not apparent throughout *Sphagnum* species. This study was devised to firstly, to create a competent protocol incorporating nuclei extraction and flow cytometry that would be relatively easy to follow and efficient. Secondly the study wanted to gain a wider understanding of ploidy levels in micropropagated material for better application to *Sphagnum* farming practices. Thirdly the potential of synthetic polypoidal inducement using a known herbicide was to be investigated to attempt to achieve a polypoidal state in diploid species or a general increase in ploidy in haploid species. Lastly, species from various origin where to be assessed to distinguish regional difference in species across Europe.

This study offers a comprehensive protocol including nuclei extraction, flow cytometric preparation, and an acquisitional gating strategy to ascertain *Sphagnum* nuclei with low background debris. Nine species of micropropagated *Sphagnum* species where analysed and recorded. Standards have been outlined in haploid and diploid species which can be used as a reference when analysing *Sphagnum* material for future studies A wide screening of oryzalin induced material at various thresholds of higher oryzalin (120 μm) displayed characteristics initiating large fluctuations in in relative fluorescence when compared to a non-induced standard indicating that oryzalin did influence diploid specimens. Further subculturing, secondary oryzalin inoculation trials, and cell sorting techniques, could result in a whole genome duplication from diploid to polyploid. Regional diversity was seen in *S. fallax* with a diploid variant identified from a German source but may indicate fixed heterozygosity due to the lack of natural polyploid populations. This study provided the first whole genome duplication (GWD) using oryzalin In *S. squarrosum* resulting in a diploid variant.

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General Introduction

The *Sphagnum L.* genus is widely regarded as the 'keystone' for the recovery of degraded ombrotrophic peatlands (Rochefort 2000). Due to Its ability to rehabilitate declining peatbogs a rapid demand for the supply of the genus has become apparent (Caporn et al., 2018). The rise in demand of *Sphagnum* proves consequential of the diminishing rate of intact peat bogs and the rising annual extraction and usage of horticultural peat. It was estimated that 20,000,000 m^3 of peat is used in the EU annually (Altmann, 2008). This vast degree of extraction has inevitably left only 640,000 ha (22%) of the UK's peatbogs remaining in near perfect condition which remain to act as a significant net for CO_2 (1,800 kt CO 2 yr 1) (Evans et al., 2017). Unfortunately, most UK peatlands fall under the carbon source GHG (Green House Gas) emissions contributing to 15% of total emissions in the UK in 2017 (Evans et al 2017). Due to the alarming rate of increase in global GHG emission, (12% higher in 2019 than 2010 and 54% increase since 1990, IPCC, 2023), and the coinciding scale of peatland degradation, GHG neutral alternatives to peat were developed and coined as 'carbon farming'. Sphagnum biomass farming has been outlined to be an effective and successful alternative to conventional peat-based substrate in professional horticulture (Wichmann et al., 2015), and has been continually researched by (BeadaMoss, UK). There are host of methods that can enhance sphagnum farming yields and plasticity one of them being development of Sphagnum genetics. Mutating plant genetics is widely documented for agronomic and horticultural benefit (Jaskani et a., 2005; Chen et al., 2006 Contreras et al., 2007). The reoccurring similarity in mutating species is synthetic chemical inducement creating multi chromosomal numbers known as polyploidy. The potential of unlocking higher levels of ploidy in *Sphagnum* could enhance sphagnum farming and peatland rejuvenation alike.

0.1 Peatland Significance and Decline

Peatlands are the largest long-term carbon store in the terrestrial biosphere, whilst being one the most important ecosystems in terms of feedbacks to global climate change (Bridgham et al., 2008). Non-degraded peatlands host a large accumulation of terrestrial organic matter fixed from the atmosphere by photosynthesis which in turn leads to a large accumulation of carbon rich peat (Page and Baird, 2016). Peatlands play a vital role in ecosystem maintenance minimalizing risks of flooding via the means of regulating water flows and provide local products that sustain local economies (IUCN, 2019). Not only this they provide an abundance of historical information via paleoenvironmental records and hydroclimatic sensitivity. Analysis of the anaerobic top layer of peat conceals a long lineage of climatic information due to its substantial limiting decay and preservation of material (Jackson and Charman 2010). The numerous benefits that peatlands possess make them an essential environment to preserve and maintain. Adversely, this has not been the widely accepted outlook on said environments. Peatlands have become progressively manipulated through generations by the means of multiple anthropogenic past times such as fuel, agriculture, and forestry (Gorham et al., 2002). By 2004 more than 50% of peatland area in Europe had been converted mainly into agricultural lands (Bryne et al., 2004). Total land use change overall has led to a 15% global peatland habitat loss in a century (Barthelmes 2016).

0.2 Sphagnum Moss and Peatland Restoration

Year upon year peatland restoration programs is increasingly being integrated around the world (CBD, 2014). Important moorland sites are generally in unfavorable conditions due to the absence of *sphagnum* species within vegetated areas (Carroll et al., 2009). This is due to *Sphagnum* possessing morphological, anatomical, and physiological traits that help alter their environment by forming nutrient-poor, heat-insulating, and slowly permeable peat (Rochefort 2000). S*phagnum* farming (Gaudig., 2014) is one of the central restoration programs used throughout Europe. Sphagnum farming refers to the cultivation of sustainable sphagnum biomass used for growing substrates and reintroduction material for peatland projects (Pouliot, et al, 2015). Sphagnum farming has been outlined to work effectively on a large scale across the world (Pouliot et al., 2015; Gaudig et al., 2017) and is closely associated with micropropagation. Micropropagation involves growing sterile material from selected genotype *In Vitro* tissue culture (Debergh et al., 1991). Micropropagation involves the use of a controlled laboratory environment which are optimum conditions to carry out ploidy investigations due to the measures of sterility put in place and *Sphagnum* being in an *In Vitro* stage. Specifically increasing the level of ploidy (sets of chromosomes) has been widely conducted with In *Vitro* cultures and have often found to be the most effective stage of growth to inducing this phenomenon (Hannweg et al., 2016).

0.3 What is Ploidy

Polyploidy refers to the cellular state in which a species has more than two sets of chromosomes per nucleus. This development has proven to be an essential progression for plant evolution and diversification (Soltis et al. 2009). Polyploidy commonly occurs via two means, firstly by the means of a multiple sets of chromosomes deriving from one species (autopolyploid), the second via the means of multiple sets of chromosomes comprised of two or more different species of origin via hybridization (allopolyploid) (Stebbins 1950). In addition to naturally occurring polyploidy, synthetic polyploids can be induced by incorporating chemicals such as oryzalin which interfere with cell division resulting in genome doubling (Dhooghe et al., 2011). Inducement of polyploidy via Oryzalin treatment is well documented across a range of plant species, including angiosperms (Morejohn 1987), Gymnosperms (Contreras, 2010) and even in Bryophytes (Alix et al., 2017). Furthermore, artificial inducement of polyploidy in plants is a well-established process in a host of different plant species often enhancing agronomic traits creating higher physiological and morphological plasticity (Chung et al.,2017; Wei et al., 2018).

Polyploidy has established evolutionary phenotypic traits in plants such as creating compact growing phenotypes establishing denser and more robust individuals than their diploid counterparts (Danaeghel et al., 2018). Secondly the duplication of the genome results in an increased number of alleles in polyploids which can have a protective effect in removing damaging recessive mutations and genotoxicity (Comai, 2005), whilst ensuring against the loss of contribution to the gene pool. Thirdly, polyploid individuals are host to an increased level of heterosis (Improved or increased function of any biological quality in an offspring). Polyploidy provides initiating properties of increased robustness, increasing tolerance to invasive species whilst resulting in higher rates of fertility outlining a transgressive performance over its progenitor material (Birchler *et al*., 2019). Lastly, duplicated genes can evolve to assume new gene function (Gottlieb, 2003), increasing the degree of flexibility in the genome (Levin, 2002) allowing individuals to be more susceptible to new niches as stress resistant and drought tolerance are characteristic of polypoidal species (Van Leare et al., 2010).

0.4 Ploidy in Sphagnum

Ploidy levels in *Sphagnum* are found to be either haploid (n=19) or diploid (n=38) across the UK (Anderson and Smith, 1980). *Sphagnum has* been found naturally as an allopolyploid in other parts of the world as a result of hybridization between taxonomically similar *Sphagnum* species (Ricca and Shaw, 2009, Behling et al., 2019). Allopolyploid hybridization is relatively common within the *Sphagnum* genus (Ricca and Shaw, 2010). The hybridization of *Sphagnum* x *falcatulum* and *Sphagnum* x plantifolium (Karlin, 2014) provide evidence of a triploid nature and are consistently found naturally through the Holarctic realms, stemming from three monoploid genomes (Karlin, 2017, Alix *et al*., 2017). Of the 36 documented natural *Sphagnum* allopolyploids confirmed (Meleshko et al., 2018), none are found in the UK and are predominately found in the Southern Hemisphere are predominately

found in the southern hemisphere. Allotriploid *Sphagnum* individuals in the Southern Island of New Zealand are found to be the most predominant species due to a competitive advantage over diploid individuals allowing for inhabitancy over wider niches (Behling et al., 2019). There is no evidence suggesting allopolyploidal populations in the UK, although recently the first ever documented polypoidal *Sphagnum* species (*Sphagnum* x *lydiae)* of northern hemisphere origin has been identified in tundra vegetation of northern Russia (Kyrkjeeide et al., 2019). *Sphagnum* has not been identified in the natural environment as an autopolyploid, though this does not entirely rule out the possibility of its occurrence.

Many of these naturally occurring allopolyploid individuals exhibit dominance in the natural environments in which the species occur. It may be that polypoidal Sphagnum could accelerate the progress of peatland rejuvenation in the UK in terms of establishment and continuity. In addition to the naturally occurring polypoidal species described above it may be possible to induce ploidy, using for example Oryzalin. However, there is little research into *artificially* inducing polyploidy in *Sphagnum* and other bryophytes, yet the potential application of a higher genetic development (polyploidy) via the means of oryzalin inducement in *Sphagnum* possesses many potential benefits which could be critical for carbon sequestration and agronomic benefit. These benefits include creating individuals of increased vigor which are less susceptible to being over dominated in their habitat by invasive species such as weeds. The resilience of the *Sphagnum* will allow for consistency and optimum functionality of peatland sequestration whilst aiding the re-establishment of native fauna and flora (Lindsey, Birnie and Clough, 2016). Secondly, the ability of gene redundancy of a polyploid individual through masking of recessive alleles, diversifies gene function by altering redundant copies (Comai, 2005). This allows species to create environmental tolerances resulting in enhanced competitive abilities and niche expansion (Visger et al., 2016). Potential habitat expansion in *Sphagnum* moss could allow for acidification and waterlogging in degraded areas of peatland across the UK, helping regain a more near natural state in the damaged environment's that comprise 80% of the UK's peatland habitats (Bain et al., 2011).

0.5 Measuring Ploidy

In order to study polyploidy in plants, reliable and consistent methods to quantify and measure levels of ploidy are required. Quantification of ploidy is generally carried out via quantifying DNA within the nuclei using fluorescence flow-cytometry. This procedure historically has been used for mammalian cells and clinical procedures (Betters, 2015) but has been adapted for plant nuclei (Dolezal, 2007), and the procedure has even been adapted specifically for bryophytes (Bainard and Newmaster, 2010). The machine exposes nuclei to a series of excitation lights measuring the level of fluorescence (Bohanec, 2003). To measure fluorescence a nucleus is extracted and a fluorochrome is used (e.g., Propidium Iodide (PI) or DAPI) which binds to the DNA within the nuclei (Bainard, 2010; Heck *et al*., 2020). The higher the level of fluorescence, the higher the DNA content (Todd et al., 2018). The output of flow cytometry is often represented in a histogram format distinguishing ploidy pattern based on the DNA content of the populations present.

Direct measurements of DNA are typically supplemented by visualisation of phenotypic changes, such as length and width and diameter of branches (Regalado et al., 2017). Image scanning techniques are often run parallel to phenotypic assessments providing measurements of area for desired regions of the plant (Fahlgreen et al., 2015). Imagining is often used particularly to determine growth differentiation in capitulum of *Sphagnum* (Heck *et al*., 2020).

0.6 Previous work

Previous work at Manchester Metropolitan University investigated *Sphagnum* species induced with oryzalin. The study was comprised of three main sections - (1) phenotypic growth analysis (2) pilot study/method refinement and (3) final flow cytometry study. Four species were used for the study, two of which were haploid species (*S. fallax*, *S. capillifolium*) and two diploids (*S. palustre, S. papillosum*), which were oryzalin-treated to induce polyploidy.

The phenotypic growth analysis did not initially outline the visual growth changes anticipated when comparing the control material to the induced material. *S. fallax* (Induced) phenotypic traits deteriorated when compared to the level of growth in the control material. *S. palustre* (Induced) did not show the level of stunted growth as *S. fallax* however did have marginally smaller levels of growth than the control material. The oryzalin proved to have a more prominent effect in *S. capillifolium* and *S .papillosum* as both species displayed an significantly enhanced level of growth over a large number of the traits analysed when compared to their control counterparts. Haploid (*S. fallax, S. capillifolium)* and diploid species (*S. palustre, S. papillosum)* had notably different traits such as number of capitula and area (sample size) which where both higher in the diploid species.

The initial pilot study outlined a clear basis for a haploid external control *P. sativum* and aided in adaptation of the nuclei isolation protocol to achieve debris free samples which provided substantially better results when analysed by flow cytometry. However, the *S. palustre* samples analysed ploidy levels could not be determined due to low nuclei count, low fluorescence, and broad peaks. It was outlined that *Sphagnum* had a low DNA content which truly showed when trying to get measurable readings in the flow cytometry stage. Low yields of nuclei have been evident in *Sphagnum* analysis particularly and has been shown to have a small genome species wide (0.42 pg) (Temsch, 1998) and have often shown counts lower than 200 nuclei when analysed using Flow Cytometry (Heck et al., 2020) making it problematic to conduct statistical analysis as 1000 nuclei are conventionally required (Dolezel, 2007). Refinement was undertaken by conducting analysis using fluorescence microscopy which deciphered the location of nuclei populations in the preliminary stages of the study. Microscopy outlined that the material being used was aged beyond the point of analysis as a large portion of the chlorophyll in the samples was black. The developments made from this called for the protocol to use young actively growing material extracting capitulum material exclusively for flow cytometry analysis.

The refined flow cytometry showed initial improvements from the pilot study. Histogram peaks identified were well established with sufficient nuclei counts for statistical analysis outlining minimal debris. The herbicide induced species *S. capillifolium* and *S. papillosum* outlined lower nuclei counts than the control suggesting that oryzalin had been effective at inhibiting the division of the cells in both induced species. *S. capillifolium* outlined little difference in terms of fluorescence to the control material. *S. papillosum* however outlined substantial differences from the control, three different population peaks where consistently apparent in the induced *S. papillosum* suggesting some form of polyploidy had occurred. Due to the low count of nuclei and small multiple peaks it was suggested that *S. papillosum* had gone through a stage of endoreduplication resulting in endopolyploidal (A polypoidal state in which chromosomes have divided repeatedly without subsequent division of the nucleus or cell) samples. The primary issue with the refined flow cytometry was still the fact that fluorescence was still not functioning accordingly making it difficult to determine what state of ploidy was identified in either the induced material or the control material. Due to the low level of fluorescence a non-conventional graphical representation had to be used (Bi-Exponential). Using this output graph made the results problematic to interpret and contrast to other findings in academic literature. Due to the nature of the prior project and the lack of education of Flow Cytometry

presentable statistics where not extracted and replicable for the project at hand. Comprehensive graphical representation and microscopy work are available upon request.

Concluding on oryzalin inducement in the *Sphagnum* genus showed promise as a method to induce chromosomal change. Enhanced phenotypic traits and potential polyploidal states all appeared consequential of herbicidal incorporation. Polyploidal inducement is almost certainly species dependent and could require an entirely different set of parameters to achieve such a state regardless of how taxonomically similar species are throughout the genus. Limitational elements throughout the study resulted in an uncertain conclusion predominantly due to the statistical carry out, limited understanding of flow cytometry processes, and the limited number of replicates in species analysed.

0.7 Intended Study

Objectives

1.) **Create a competent nuclei extraction method alongside a flowcytometry output procedure comparable to external standard- (Chapter One).** This objective will create a refined nuclei extraction protocol providing interpretable flow cytometry results. A recent protocol outlined by (Heck *et al.,* 2020) displays clear nuclei fluorescence in *Sphagnum* material using a DAPI stain procedure. Fluorescence was the primary problem with the initial study, however, with a proven functional method nuclei fluorescence can occur consistently. In coherence with this an external standard of cell cycle arrest and genome sizes will be implemented against known bryophyte species. Stage two of the process proceeded after a functional method of Flow cytometry had been adopted. Micro-propagated *Sphagnum* species will then have a known level of DNA for internal standards allowing the possibility to quantify different characteristics in populations when carrying out the flow cytometry. **Outcomes:** Trial and test fluorochromes and buffers outlining one that will successfully bind to nuclei creating consistent and comparable results. Develop a comprehensive understanding of flowcytometry data presentation and output software to a degree in which the results can be compared to an external standard using Flowlogic Software.

2.) **Gain a precise understanding of ploidy levels in a various number of micro propagated species from the *Sphagnum* genus (Chapter Two).** Once all parameters are in place, non-induced material can be analysed in full. Non induced material will be ran first as there is reference to known levels of ploidy which will aid in validating the technique and provide a base line to whereabouts haploid and diploid populations should be apparent. At this point there will be every means to clearly see the difference in levels of ploidy in haploid and diploid species by having a comprehensive understanding of how the flow cytometry graphical output translates. **Outcomes:** A competent base understanding of the levels of ploidy in micro propagated material in a wide variety of *Sphagnum* species. A clear difference will be seen in population and confirm channel volumes for nuclei populations of different number of chromosomes.

3.) **Undertake a wide screening of oryzalin induced material at various thresholds of exposure outlining any potential enhanced level of ploidy (Chapter Three).** This objective will be carried out on the induced material as a strong basis will have been formulated on how different levels of ploidy can be identified on the flow output. The combination of data from the flow with the Flow logic output software will provide highly interpretable results and a direct conclusion if oryzalin has proved successful in inducing a polypoidal state. **Outcomes:** Screening of a host of different oryzalin induced species at a range of different exposures to work out the optimum exposure concentration for each individual species. Determine if oryzalin has induced an endopolyploidal state in diploid treated material.

Chapter One

Method Development- Nucleic Extraction and Flowcytometry Analysis

Introduction

1.1 Acquisition of Sample Material

One of the primary objectives outlined in this study incorporates the collection of sample material from a non-wild scenario. One focus of the project was to investigate levels of ploidy in *Sphagnum* material grown in a novel laboratory condition known as micropropagation. Micropropagation itself is an asexual reproductive procedure removing small quantities of somatic tissue from a mother plant to maintain desirable genotypes or provide sustainable, sterile specimens in commercial horticulture (Bohjwani and Dantu, 2013).

BeadaMoss Micropropagation Services contributed a multitude of healthy in vitro samples, to ascertain an understanding of chromosome levels within species grown from tissue culture. Sterilised jars containing nutrient rich agar implemented an effective method of separation and growth. Specimens undertook twelve to sixteen weeks of growth at Beadamoss at which point where either then stored in dark cold rooms to inhibit enzyme activity, heavily reducing growth, or delivered to the university for FCM analysis.

Twelve to Sixteen weeks of growth marks a critical stage in development as plant tissues once established whilst also still being in juvenility undergo more rapid cellular division in cells known as meristems (Velappan et al., 2017). Rapidly dividing cells provide a precise depiction of cell cycle function thus the mentioned growth period is prevalently used throughout academic research providing an abundance of analysable nuclei when compared to analysis using mature or dried material in flow cytometry (Voglmayr, 2000, Dolezel et al., 2007, Heck *et al.,* 2020).

The *Sphagnacae* genus encompasses a raft of individual species. Due to the wide proportion of species, it was devised that an array of species would be recorded to best reflect the wider demographic within the allotted project time frame. Suitable material would be comprised of species having a confirmed external ploidy reference standard provided by Kew Science DNA –C value Database providing information on level of ploidy (C value) and DNA amount in picograms (pg). (Plant DNA C-values Database | Royal Botanic Gardens, Kew, 2023).

Often considered to be unequivocally haploid (single set of chromosomes) or diploid (two sets of chromosomes), (Temsch et al., 1998, Greilhuber *et al.,* 2003) both species of a haploid and diploid nature were investigated. Throughout the method development stage two species were used to establish a basis of haploid (*S. fallax*) and diploid (*S. palustre*). Further analysis conducted on other species where extrapolated from the parameters created with these two species.

Appropriate conditions where vital in the proceedings from the arrival of specimens from BeadaMoss. It was important that the integrity of the samples remained, eliminating any potential contamination, and ascertaining stable continual growth. Replicating atmospheric conditions created at the samples prior location was fulfilled by a (Percival AR-66L) growth chamber. This allowed for accuracy of light in the growth chamber was supplementary tested using a Skye Instruments LTD light measurement gauge providing an 8.72 x10 µmol reading cohering with Micropropagation Services guidelines of 5-10 x10µmol and other successful normal light level growth studies in artificial conditions (Schumann et al 2017, Glime, 2017).

1.2 Protocol Development

Understanding genome sizes and chromosome sets in plants was revolutionised by the application of flow cytometry. Traditionally flow cytometry was developed to investigate mammalian cells in immunobiology (Picot et al., 2012). However, adaptions in protocols have made FCM an applicational success story in plant-based cells for over four decades, now being one of the most powerful and widely adopted methods to assesses DNA content and ploidy (Vrana et al., 2022).

Protocols created by (Dolezel et al., 2007) outlined a rigorous procedure how to successfully isolate cells of interest (nuclei) from plant material ensued by reliable methods of how to appropriately analyse, quantify, and troubleshoot any outcome that may occur whilst undergoing FCM. Post (Dolezel et al.,2007) a protocol released by (Bainard and Newmaster 2010) refined and localised FCM for bryophytes specifically investigating endopolyploidy. A form of endoreduplication in which genetic material is cloned without going through mitoses resulting in bigger cell sizes (Leach and Dodsworth 2017). In which *Sphagnum* was analysed but not discussed in volume due to it not exhibiting endoreduplication. (Heck et al.,2020) published a study in part outlining a procedure for *Sphagnum* nuclei however due to differences in machinery it would have made difficult to follow this protocol.

Dolezel outlines the importance of trialing several protocols to tailor, stemming from using an array of buffers, dyes, chopping procedures and quantity of sample material. All variables have the potential to drastically change several factors including, nuclei yield, debris in the samples, and level of fluorescence. Following this, a need for a competent protocol for nuclei extraction was a clear starting point for the project. The protocol in question would follow guidelines already outlined by (Dolezel et al., 2007, Bainard and Newmaster, 2010) with changes being made incrementally.

1.3 Overview of Isolation Procedure and Fluorochrome Staining

A nuclear isolation in FCM is a series of stages devised around extracting and preserving cells such as nuclei, whilst facilitating the breakdown of cytoplasmic debris and reducing potential aggregation of cells in a liquid suspension (Loureio, 2006). Moreover, a series of variables can determine the abundance of nuclei populations, debris, and the level of fluorescence when ran using FCM machinery. There are many protocol variants of nuclear isolation in plant cell flow cytometry and optimization of each is often species specific.

The process of nucleic isolation begins by understanding how much plant material is required to provide a substantial yield of nuclei. The weight of material used from an individual sample for analysis corresponds to the DNA content found within the cells of the species (Sliwinska et al.,2021). In model reference species such as *P. sativum* (Common Pea Plant) DNA is in large abundance due to its large genome size (DNA pg =4.78) and does not require large volumes of sample material. In contrast, *Sphagnum* species hold lower quantities of genetic material (*S. squarrosum,* DNA pg=0.46), this dictates that a larger quantity of sample material is needed to extract sufficient nuclei for statistical analysis as most mosses are defined by small genomes (Bainard 2020). Once an estimated quantity of sample material is decided, precise weights can be determined when carrying out FCM.

Once considerations to weight optimums are finalised, a subsequent isolation buffer is required to lyse cell populations of interest. Isolation buffers designed for plants differ from buffers created for mammalian cell cultures, one of the most prominent differences being the extensive variety of buffers developed for plant cell cultures. The diversity of plant tissues and their chemical compositions necessitates specific buffers for individual species (Sadhu et al.,2016) resulting in over twenty-eight different buffers currently registered in the FLOWer database (Loureio et al.,2016). Consequentially a series of buffer testing is required to identify which buffer composition facilitates the release of intact nuclei populations, protects DNA, and allows for successful stoichiometric staining (Coba de la Peña & Sánchez-Moreiras, 2018). The volume of buffer solution is also a large factor when trying to carry out the next step, the chopping procedure.

An essential aspect to successful nuclei isolation is carrying out a competent chopping of sample material. Once applied, the buffer begins to break down unwanted debris but is only facilitated by the physical chopping of the sample. The chopping itself is empirical to the specimen (Dolezel., 2007). Factors effecting the yield of nuclei included rigorousness, and duration of chopping. Quality and temperature of chopping apparatuses can also be critical to gaining a high-quality sample. Chopping in plant material is often carried out quickly but not intensely to avoid the release of cytosolic compounds (Wang, 2015) as presence of such compounds when using DNA binding dye have been seen to reduce fluorescence by 20% (Noirot et al., 2000,2001,2003). Sample chopping is followed by homogenising using a pipette, a volume of the homogenate is then passed through a nylon mesh filter to remove any larger debris which could damage the flow cytometer during analysis. Samples are then incubated on ice for a duration of time after which a stoichiometric dye is applied imminently alongside RNase (a) before analysis.

Stoichiometric staining initiates the final stages of the isolation procedure. Fluorochromes can be used to bind to host of different nucleic acids and proteins to analyse ploidy, cell proliferation, and apoptosis (Sharrow 2001). The dye itself allows for visual representation of populations of interest being excited at a certain wavelength of light and emitting at another wavelength. Staining dyes used in plant species commonly are Propidium Iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI) (Dolezel, 2007, Heck et al., 2020, Bainard and Newmaster, 20220, Temsch, 2022). In most cases cells must be fixed or permeabilized to facilitate DNA binding dyes, which in turn removes all live cells but provides histogram profiles with lower coefficient variants allowing for greater certainty of individual populations when carrying out FCM analysis.

Methods

1.4 Material Quantity

Understanding the threshold of nuclei to debris would dictate the optimum weight of material to use for analysis whilst providing clear populations that are possible to separate from unwanted material. Sample material analysed was rapidly growing young material (8-12 weeks of growth) being extracted on the same day of analysis (Dolezel et al,2007). It was already understood from prior morphological analysis by fluorescence microscopy that nuclei distributed in *Sphagnum* are most abundant in the capitula, responsible for creating sporophytes. For this reason, sample material extracted was taken entirely from *Sphagnum* capitula.

Capitulum material was removed from the sample jars using sterile forceps to inhibit potential contamination, measured on a four decimal balance and placed into a petri dish. (Dolezel 2007, Bainard and Newmaster et al.,2010) protocols guidelines suggested material should be between 10 milligrams and 20 milligrams. Due to the nature of low genetic quantities in *Sphagnum* the higher guideline of 20mg was selected. A selection of both haploid S*. fallax* and diploid *S. Palustre* were tested at 20mg, 40 mg, 80 mg and 120 mg weights respectively.

1.5 Isolation Buffers

The effect of nuclear isolation buffers on FCM performance were tested in partnership with the University of York whilst undertaking an intensive flowcytometry training course. The buffers included the LB01 buffer (Dolezel et al.,1989), Tris MgCl2 (Pfosser et al.,1995) and Galbraith (Galbraith 1983). *Sphagnum* of haploid (*S. fallax)* and diploid (*S. palustre*) where tested with each buffer and compared to a diploid reference *P. satvium* (Common Pea Pant). 40mg of sample material was chopped and received 1ml of buffer post chopping. PI and Rnase (a) were used simultaneously at 150ug ml and 50ugml respectively (Dolezel et al.,2007). Samples where incubated on ice for a further 10 minutes before being analysed on a Beckman Coulter cytoFlex Flow Cytometer. All buffers were stored at - 20°C and used at ice-cold temperatures to inhibit cell shock and the breakdown of the nuclei of interest.

1.6 Fluorochrome Type

Following the successful binding of using PI (Propidium Iodide) in the trials with The University of York it became the main dye used throughout the study. Alongside using PI, a small trial using a variant of (Hoeschtt 33342) named 'NucBlue' was used in attempt to streamline the processing a high throughput of samples. Nucblue a relativity recent fluorochrome had not been used vastly throughout plant flowcytometry and almost non-existent in bryophyte FCM. The fluorochrome is excited in UV light at 360 μm and emitted in blue light at 460 μm (Thermofisher.com), the implications of this being a whole new spectrum of light to observe *Sphagnum* nuclei as in contrast PI is detected much higher in the light spectrum at 610 μm. An alternate spectrum of light to analyse nuclei could potentially provide greater yields with less residual debris.

Nucblue also removed the requirement of pipetting fluorochrome and replaced with a drop bottle that required two drops per ml. If successful in providing competent results the usage of this alternative fluorochrome could streamline the process of analysis in a high through put of samples. Nucblue trials were chopped and buffered using the same protocol as the propidium Iodide trials. Samples stained with Nucblue did not receive RNase(a) as the dye binds to all nucleobases as opposed to PI only binding to A-T rich regions (Dolezel, 2007). Three replicates of *S. fallax* and *S. palustre* where stained with PI then compared to three replicates stained with Nucblue. Samples were assessed by C- values and relative standard error, compared to an external reference provided by Kew C-Values Database.

1.7 Fluorochrome Staining and Duration

Fluorochrome staining duration and staining concentrations were not tested individually as (Bainard et al., 2010) highlighted an optimum staining duration at 10 minutes as entire staining saturation could occur quicker in species with smaller genomes. Concentrations of PI were shown to have up to a 14% difference on 1-C (pg) values in bryophytes, with lower saturation between 10-50 $\mu g\ ml^{-1}$ did not reach an entire saturation of all cells. Higher concentrations such as 100-150$\mu g\ ml^{-1}$ allowed for a full saturation to occur yielding the greatest number of fluorescing nuclei. Short periods of staining and high concentrations of PI were used to replicate this. Samples received 150$\mu g\ ml^{-}$PI before being incubated on ice for 10 minutes in total darkness.

1.8 Chopping Trials

Due to chopping being empirical it poised for several factors to be considered. The successful chopping in the buffer trials suggested that a non-rigorous fast chopping eased nuclei into suspension without obliterating the structure of the cell. To further quantify this measurement, chopping variables considered included timed chops between 10 seconds and 2 minutes. A series of soft to hard rigorousness chops where also included. Razor blades were stored at ice cold temperature and only used on one sample.

1.9 Flowcytometry Analysis

Sphagnum samples were analysed using a BD FACSCelesta (BD Biosciences, New Jersey, USA) equipped with a blue solid-state laser tuned to 20mW. Operating at an excitation wavelength of 488 nm expected emission was around 630 nm (BD Spectrum Viewer). Instrumentation was calibrated before each use using 5um calibration beads (BD Biosciences, New Jersey, USA). Parameters recorded for each samples included FSC (forward scatter-cell size), SSC (Side Scatter-cell complexity).

Due to chopping in the nuclei isolation procedure, separation of nuclei from debris is made impossible by the conventional method of plotting cell size (FSC) vs cell complexity (SSC). Instead, the channel, PE-CF594 was used to separate nuclei from debris by plotting against FSC-A (see Fig.1) FSC-A was plotted against FSC-H to as a means of doublet discrimination as some nuclei can coagulate and give the reading of one individual with a higher rate of fluorescence (Stadinski and Huseby,2020) eradicating the potential of false ploidy readings. A second and tertiary, doublet discrimination where created used to further nullify false ploidy readings using SSC-A vs SSC-H, and PE-A vs PE-H. Finally, isolated nuclei data was collected in the PE channel (578 nm) as PI channel was not optional on the instrument.

Sample and standard peaks of interest had nuclei number and co-efficient variant (CV) recorded using a series of gating functions using Flow logic Flow Cytometry Analysis Software (Version 8.6, 2022). To determine the mean peak position and CV of the histogram peaks, regions were created between the peak start and the peak end. Literature would suggest commonly that peaks read over 1000 nuclei and acceptable peak CV's fall between 3-5% (Dolezel, 2007, Tomaszweska, et al 2021). Due to the *Sphagnum* genus 's small genome sizes, methodology used does not always provide the outcome anticipated (Bainard et al., 2010). Reporting's would suggest that low nuclei across bryophytes collectively can be close to the resolution threshold of most cytometers making them only just visible on the instrument. (Voglmayr, 2007).

Flow cytometric analysis for the weight and buffer performances were calculated by taking the nuclei count and CV of each histogram peak to create a relative standard error (RSE) (Bainard et al., 2010). Formulated (RSE=SE/Mean). The ability to factor nuclei counts alongside CV's provides insight when data would be conventionally disregarded due to high CV's.

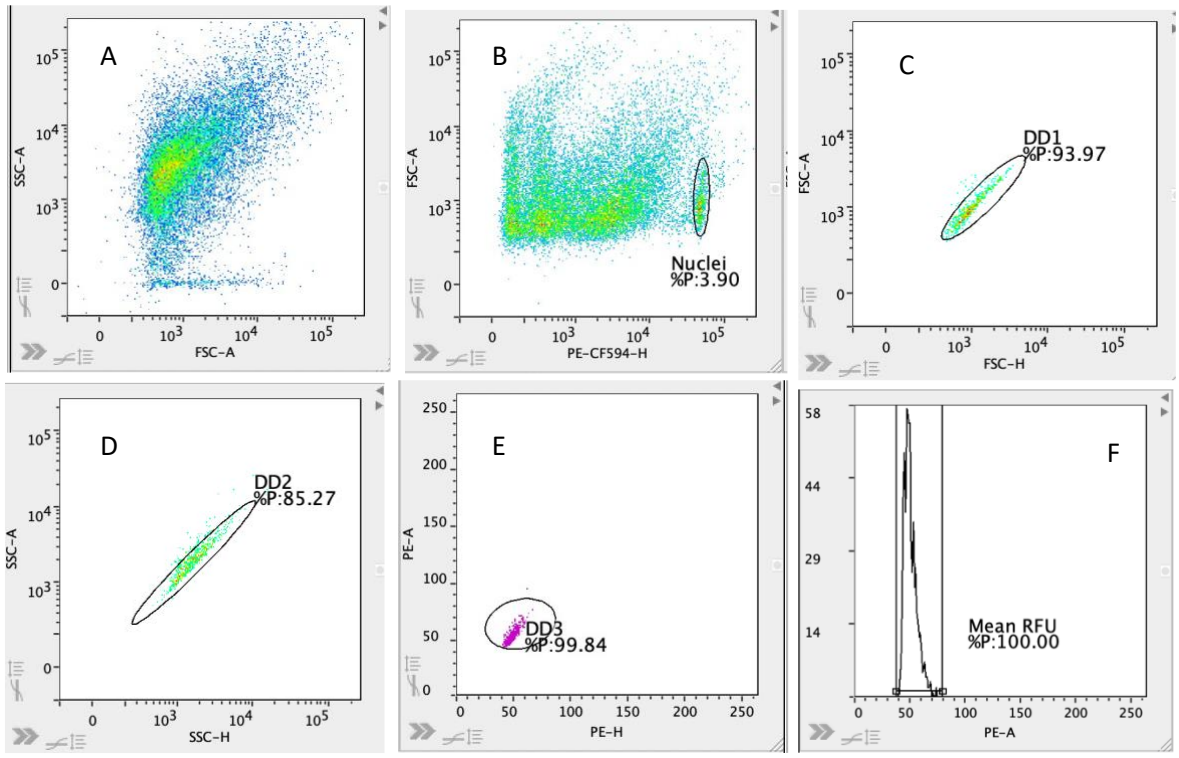


Figure 1 Acquisitional linear gating profile of a standard haploid S. fallax, (A). Scattergram of ungated SSC-A (complexity) vs FSC-A (size) population of interest unidentifiable. (B) FSC-A vs PE-CF594 with separation of nuclei from debris gate %p- indiciates percentage of total events comprised within the gate. (C) DD1- Doublet discrimination of cell size (FSC-A vs FCS-H) data not following proportional increase gated out. (D) DD2- Doublet discrimination of cell complexity (SSC-A vs SSC-H) data not following proportional increase gated out (E)DD3- Doublet discrimination of nuclei in channel of data recording (PE-A vs PE-H). (F)- Histogram of channel fluorescence (PE-A) against count of nuclei, (Mean RFU) a histogram marker of interest providing the mean channel volume of the histogram.

Nuclear genome sizes/Ploidy C-values were calculated using the formulas outlined by (Dolezel et al., 1992). C-values are a measurement of mean ploidy level in the samples that allow for estimation quantifications of both DNA in picograms and ploidy values (Engelen and Eigeles, et al., 2000) As mosses are haplophasic in gametophytic material (One set of complete chromosome complement) (Bainard et al., 2010) the 1C value is measured against a 2C Standard value.

$$\text{Sample 2C value(DNA pg or Mbp)} = \text{Reference 2C value} \times \frac{\text{sample 2C mean peak position}}{\text{reference 2C mean peak position}}.$$

$$\text{Sample ploidy(integer)} = \text{Reference ploidy} \times \frac{\text{mean position of the } G_1 \text{ sample peak}}{\text{mean position of the } G_1 \text{ reference peak}}.$$

Figure 2. Estimation formulae, for genome size (DNApg) and Ploidy (C-value) (Dolezel et al., 2007)

Results

1.10 Weight Tests

All weight of material tested provided varying genome sizes see (Table 1). For this reason, the addition of a Kew 1C reference values were used to highlight which sample weight provided closest to the target value measured in Picograms (pg) commonly referred to as a 1C-value.

The Mean Relative Standard Error (RSE) is used throughout analysis to highlight sampling error as a percentage (%) relative standard errors >25% should be used with caution and do not provide an appropriate level of certainty for further use in analysis.

In Table 1. *S. fallax* displayed statistically significant differences between 1C values when different sample material weight was used (P <0.0029). After all weights considered replicates using 40 mg of sample material appeared to provide the most similar 1C value to the Kew reference standard 0.42pg – 0.44pg) and the lowest RSE (2%). Replicates analysed using 80mg of sample material and 120mg alike displayed RSE values below (25%) however replicates using 20mg of material displayed (57%).

Replicates of *S. palustre* also showed statistical significance through sample weight 1C Values (P <0.00912). Similarly, to *S. fallax* 40 mg replicates produced the most relative 1C value when compared to the Kew standard (0.85 pg – 0.92 pg) with the lowest RSE value (2%). Other values in both the *S. fallax* and *S .palustre* outlined high levels of RSE

Table 1. Weight test results table analysing S. fallax (haploid) and S. palustre (diploid) in terms of estimated genome size (Mean 1C-Value in comparison to a reference mean 1c- Value) of the respective species) and relative standard error (RSE) displayed as a percentage of potential sampling error. Samples of both S. fallax and S. palustre weights were measured in sequence of 20 mg,40 mg,80 mg,120 mg. Mean value 1c value (pg) was representative of 3 replicates per sample weight type.

Sample weight	***S. fallax***			***S. palustre***		
	Analysed-Mean 1C Value (pg) + SE	**Mean RSE (%) Sample**	**Kew Reference C-Value (pg)** ***(S. fallax)***	**Analysed-Mean 1C Value (pg) + SE**	**Mean RSE (%) Sample**	**Kew Reference C-Value (pg)** ***(S. palustre)***
20 (mg)	0.37	57	0.44	1.07	24	*0.92*
40 (mg)	0.42	2		0.85	8	
80 (mg)	0.56	18		0.87	29	
120 (mg)	0.44	*19*		-	-	

Kruskal-Wallis	***S. fallax***	***S. palustre***
	P<0.0029	P<0.00912

At this stage of the project, nuclei population data was recorded in logarithmic unknowingly that analysis needed to be recorded in a linear format which is corrected in data analysis further into the project.

Inspecting the graphical representation (Fig.3 and Fig.4) of the two samples with the closest 1C values to the reference in *S. fallax* highlighted clear differences in the quality of results between both sample weights. The 40mg *S. fallax* displayed a larger intensity of acquisitional data points in the scattergram (when compared to that of the 120mg sample (A-C). The samples alike are a good indication of how little nuclei comprise of the total sample in comparison to the debris, (40 mg- 2.7% population, 120 mg 2.22% population). Both samples displayed clear separations of nuclei from debris evident by data plots appearing further along the x-axis in a higher channel volume than the clusters of debris.

The sample at 40mg weight provided higher yields of debris than that of the 120 mg sample but in turn provided a greater yield of nuclei with over 3 times the yield than that of the sample at 120 mg (B-D), (40 mg-145 nuclei 120 mg- 43 nuclei). Mean RFU gates indicate that statistical values recorded are made up of 95%> of the nuclei isolated in the scattergram polygon gates.

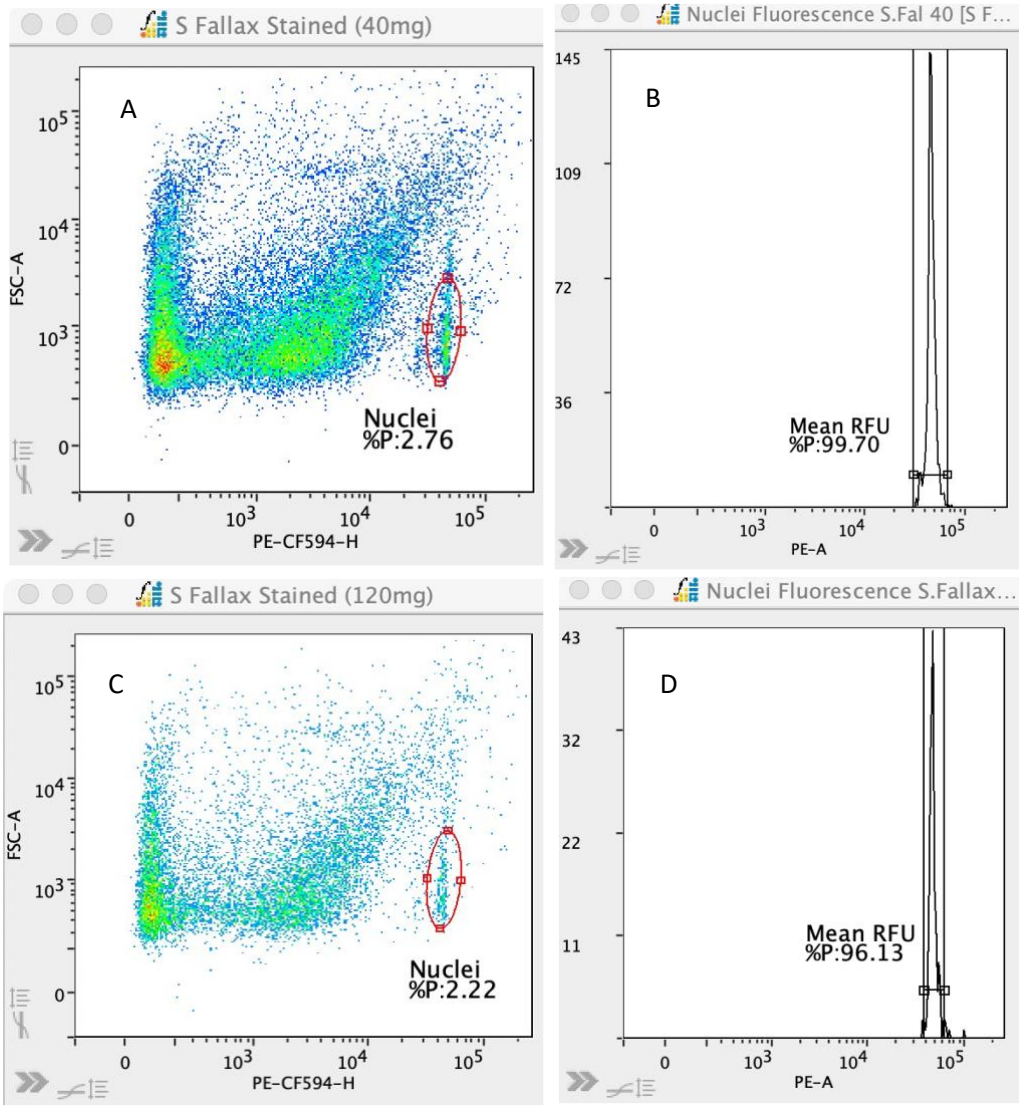


Figure 3 S. fallax 40 mg and 120 mg samples (A) Acquisitional Dot Plot of nuclei separation in the 40 mg sample (X-axis) displaying channel fluorescence. (Y-axis) displaying cell size. Nuclei percentage (%P) identified through gating. (B) Histogram of relative fluorescence in 40mg sample (X-axis) showing fluorescence of sample, (Y-axis) displaying Nuclei count. (C)) Acquisitional Dot Plot of nuclei separation in the 120mg sample (X-axis) displaying channel fluorescence. (Y-axis) displaying cell size. Nuclei percentage (%P) identified through gating. (D) Histogram of relative fluorescence in 40mg sample (X-axis) showing fluorescence of sample, (Y-axis) displaying Nuclei count.

In the diploid *S. palustre* weight trials samples of 40 mg and 80 mg provided the closest 1C values when compared to the Kew reference. Scattergram contrasts of the 40 mg and 80 mg samples (A-C) displayed considerable variation in the level of present fluorescence with a visually larger intensity of events in the 40 mg sample. This is apparent in the gated nuclei with a more properly formed population string than seen in 120mg. Nuclei population percentages were lower than that of *S. fallax* (40mg-0.97%, 120mg, 0.52%).

Contrasting histograms of fluorescence between the two samples (B-D) outline nuclei yield was higher in the 40 mg (63 events) than in the 120 mg (16 events). Nuclei yield in both *S. fallax* and *S. palustre* were 3 times higher in the 40 mg samples than other weights analysed.

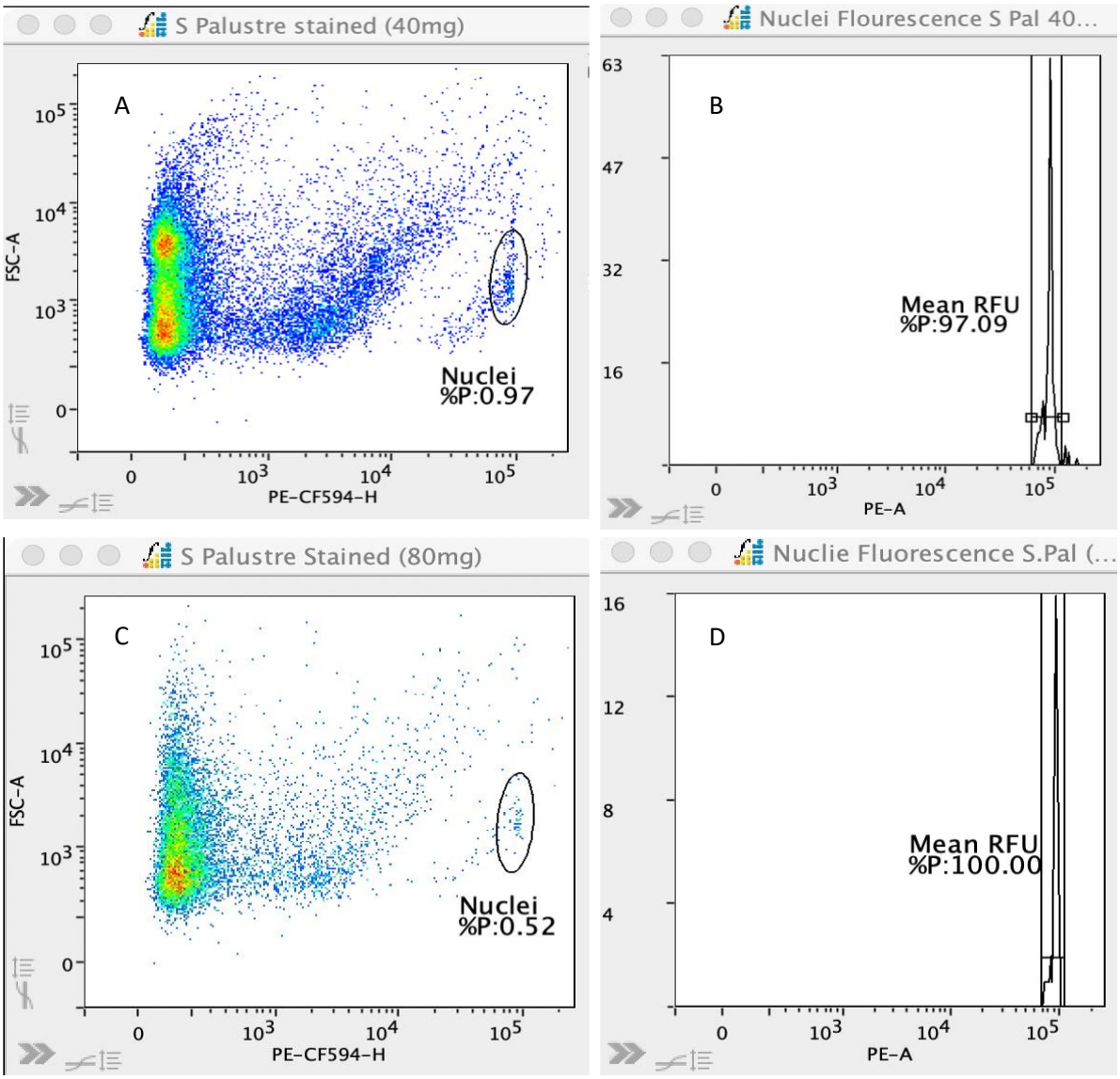


Figure 4 S. palustre 40 mg and 120 mg samples) (A) Acquisitional Scattergram of 40 mg sample outlining nuclei separating from debris. (X-axis) Channel volume (Y-axis) Size of cell. Nuclei highlighted by gated population. (B) 40 mg sample histogram of nuclei fluorescence (X-axis) relative fluorescence (Y-axis) nuclei count. (C) Acquisitional Scattergram of 40 mg sample outlining nuclei separating from debris. (X-axis) Channel volume (Y-axis) Size of cell. Nuclei highlighted by gated population. (D) 4 0mg sample histogram of nuclei fluorescence (X-axis) relative fluorescence (Y-axis) nuclei count.

The Buffer trials carried out at The University of York ploidy data was displayed on a liner scale using ($x10^4$) scale to reflect logarithmic data extracted from other channels to separate nuclei and eliminate the potential of cells coagulating when being pushed through the sample injection port. Data carried out in this study had not been carried out in the same ($x10^4$) scale and just used a conventional linear scale. This made it problematic to analyse the data obtained at The University of York in a linear fashion in FlowLogic, for this reason data collection on mean 1c values and RSE values were taken from a logarithmic format.

Table 2. Comparison of three different lysis buffers, LB01, Tris, and Galbraith effects on a haploid (S. fallax), diploid (S. palustre) and a standard (P. sativum). Performance of lysis buffers is measured in estimated genome size (Mean 1c Value pg), and a nuclei count/coefficient varience (%) metric measured in (RSE). Mean 1c values and RSE values are compared to a reference standard provided by Kew C-value Database. Mean value 1c value (pg) was representative of 3 replicates per buffer type.

Buffer	***S. fallax*** **(0.44 pg)**		***S. palustre*** **(0.92 pg)**		**Standard *P. sativum* (4.90 pg)**	
	Mean 1 c Value (pg)	**Mean 1 c RSE (%) Sample**	**Mean 1 c Value (pg)**	**Mean 1 c RSE (%) Sample**	**Mean 1 c Value (pg)**	**Mean 1 c RSE (%) Sample**
LB01	0.41	0.2	0.98	3	4.90	0.008
Tris	0.49	1.27	0.91	32	N//A	N/A
Galbraith	0.37	0.1	0.90	5	3.39	0.03

Kruskal-Wallis	***S. fallax***	***S. palustre***
	P<0.6409	P<1.5684

In Table.2 S. *fallax* the LB01 buffer provided the closest value to the standard 1c DNA content value (LB01- 0.41 pg, Standard-0.44 pg) with a low RSE value (0.002), indicating a credible value with a narrow peak margin and a high nuclei count. The Galbraith buffer provided a lower 1c value in *S. fallax* being 0.07 pg out of the reference standard being the least accurate in terms of providing the correct DNA content value of all buffers trailed when compared to a standard. The Tris buffer provided a value 0.05 pg out of the target DNA value and provided an unacceptable RSE value (1.27) due to a low nuclei count.

Lysis buffer type used in the methodological process did not display Significant difference in 1C values in *S. fallax* (P<0.6409). The *Galbraith* and LB01 buffer appeared to provide a smaller margin of error in terms of acceptance of RSE (%) than the Tris buffer. LB01 displayed the closest 1C value to the targeted reference 1C value. After utilizing the same buffers in *S. palustre* a similar outcome resulted in no statistical significance between 1C values (<P 1.5684) however did outline changes in RSE with Tris again providing the highest marginal error (32%). The LB01 buffer provided 1C values close to the reference throughout all species and replicates with consistently low RSE values. RSE appeared to be higher in the diploid *S. palustre overall*.

The *S. palustre* sample optimum buffer alternated from that of *S. fallax* with the Galbraith buffer providing a 1c value closer to the reference standard than LB01 (Ref-0.92 pg, LB01-0.98 pg, Galbraith, 0.90 pg). In fact, the Tris buffer provided the closest value (0.91 pg) however provided an unacceptable RSE value (0.32). The Galbraith buffer was just acceptable in terms of RSE value (0.05)

whilst the LB01 buffer provided the lowest RSE value (0.03).

In the P. *sativum* standard sample the LB01 buffer provided the best results replicating the exact mean 1c value seen in the reference standard (4.90 pg) The LB01 also provided the lowest RSE value (0.008). The Galbriath buffer provided a 1c value well out of the expected value (3.90 pg) but provided an acceptable RSE value (0.03). The Tris buffer performed poorly and did not provide a clear nuclei population separate from the debris.

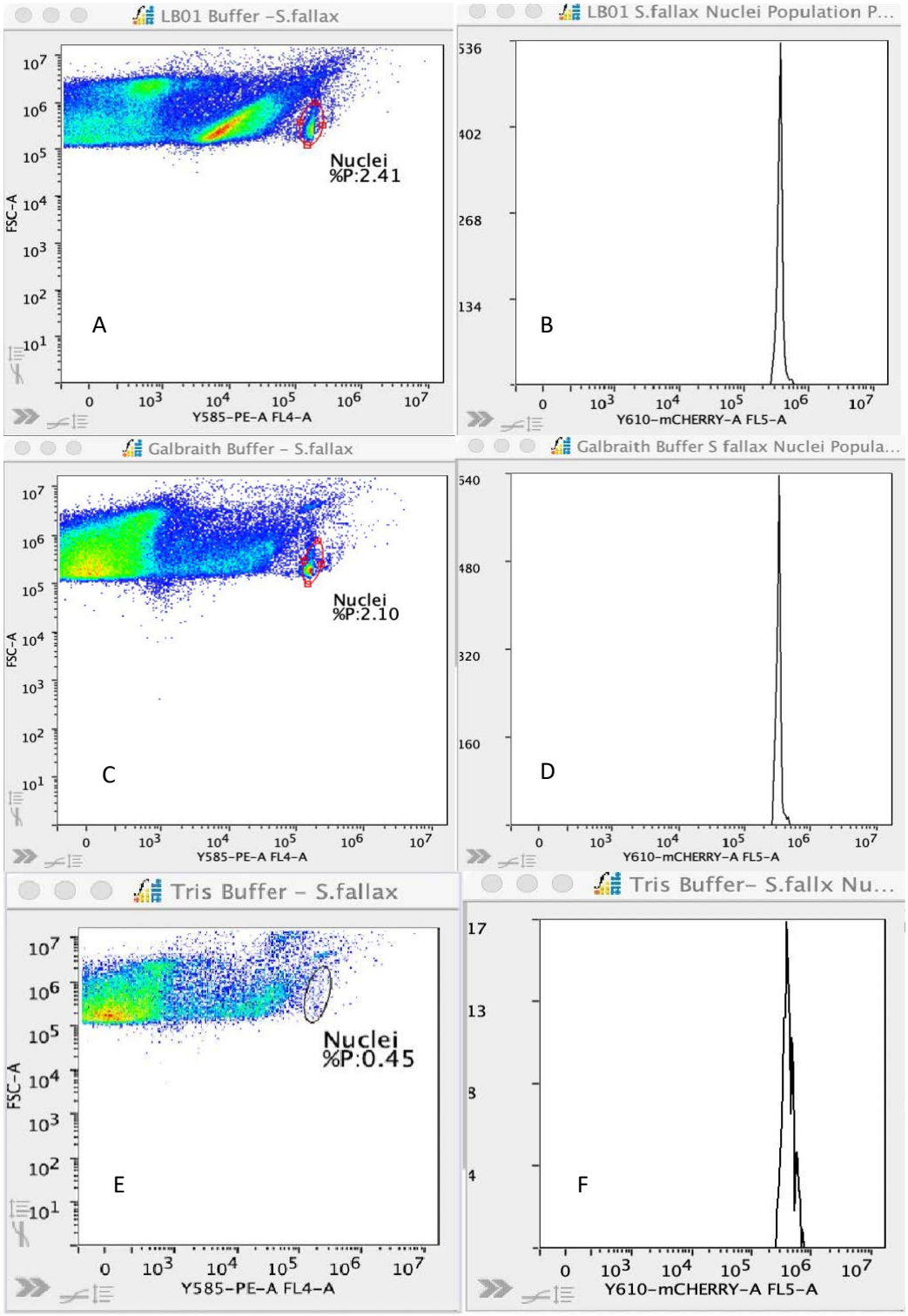


Figure 5 Scattergraph and histogram plots of three buffers (A-B LBO1) (C-D Galbraith) (E-F Tris). Scattergrams represent are plotted with Channel volume on the x-axis (Y585-PE-A-Fl4-A) and cell size on the (Y-axis). Total percentage of nuclei in the sample is represented as (Nuclei %P) and can be identified by the oval gates. Histograms are plots of channel fluorescence on the x-axis (Y610-mCHERRY-A FL5-A) against nuclei on the Y-axis.

Similarities can be drawn in terms of staining of the LB01 buffer and the Galbraith buffer (fig.5), it can be seen in scattergrams (A-C) that acquisition and separation of nuclei populations in *S. fallax* with the Galbraith buffer potentially showing a slightly more distinct separation that of LB01. This was not as apparent when the Tris buffer is applied to *S. fallax* (graphs, E-F), it's difficult to distinctly visualize a population separate from the debris so an assumed population was taken. Histogram data highlighted a similar pattern with only 17 nuclei being acquired. The other buffers used in the trial provided more than 30 times more analysable nuclei than the Tris buffer.

1.11 Fluorochrome Trials

(Table 3) Highlights considerable difference between samples stained in PI and Nucblue. The fluorochromes alike displayed observably different 1C values between haploid *S. .fallax* and *S. palustre* as anticipated. Fluorochromes did not display a significant difference in 1C values in *S. fallax* (P<0.601) but did provide statistical significance in the *S. palustre* (P<0.00902). Whilst 1C values differed greatly from the reference standard in the Nucblue stained specimens (*S. fallax + 0.08 pg, S. palustre + 0.23 pg)*, specimens stained with PI held 1C values well within the targeted reference values (<0.05pg). RSE values where considerably lower in the LB01 *S. fallax* specimens and where marginally lower in *S. palustre*.

Table 3 Comparison of the fluorochrome's Nucblue and Propidium Iodide (PI)'s mean 1c values (pg) and mean relative standard error (RSE) values in replicate samples of the haploid S. fallax and the diploid S. palustre. Mean 1C values are compared to a reference value for each species provided by Kew C-Value Database. Mean values for both dyes are representative of 3 replicates per sample type.

Sample	**NucBlue**		**Propidium Iodide (PI)**		**Kew Reference C-Value (DNApg)**
	Mean 1C Value (pg) + SE	**Mean RSE (%) Sample**	**Mean 1C Value (pg) + SE**	**Mean RSE (%) Sample**	
S. fallax (1)	0.36	5	0.42	0.9	*S. fallax* *0.44*
S. fallax (2)	0.46	26	0.42	0.7	
S. fallax (3)	0.36	8	0.42	0.9	
S. palustre (1)	0.84	*32*	0.87	*20*	*S. palustre* *0.92*
S. palustre (2)	0.69	*45*	0.88	*25*	
S. palustre (3)	-	-	0.88	*43*	

Kruskal-Wallis	***S. fallax***	***S. palustre***
	P<0.601	P<0.00902

Observations from the acquisitional scattergrams in fig.5 (A-C) highlights the differences in separation of nuclei in *S. fallax* from the debris in samples used stained with separate fluorochromes. Both highlight a density of nuclei however nuclei stained with propidium iodide would appear to have a much clearer separation from the debris. *S. fallax* nuclei stained with Nucblue appeared to comprise more of the total sample at %P3.55 in comparison to staining with PI at %P2.89. Histogram analysis (B-D) would suggest that Nucblue provided population peaks with less background noise with larger yields of nuclei (122 events) in comparison to PI (43 events).

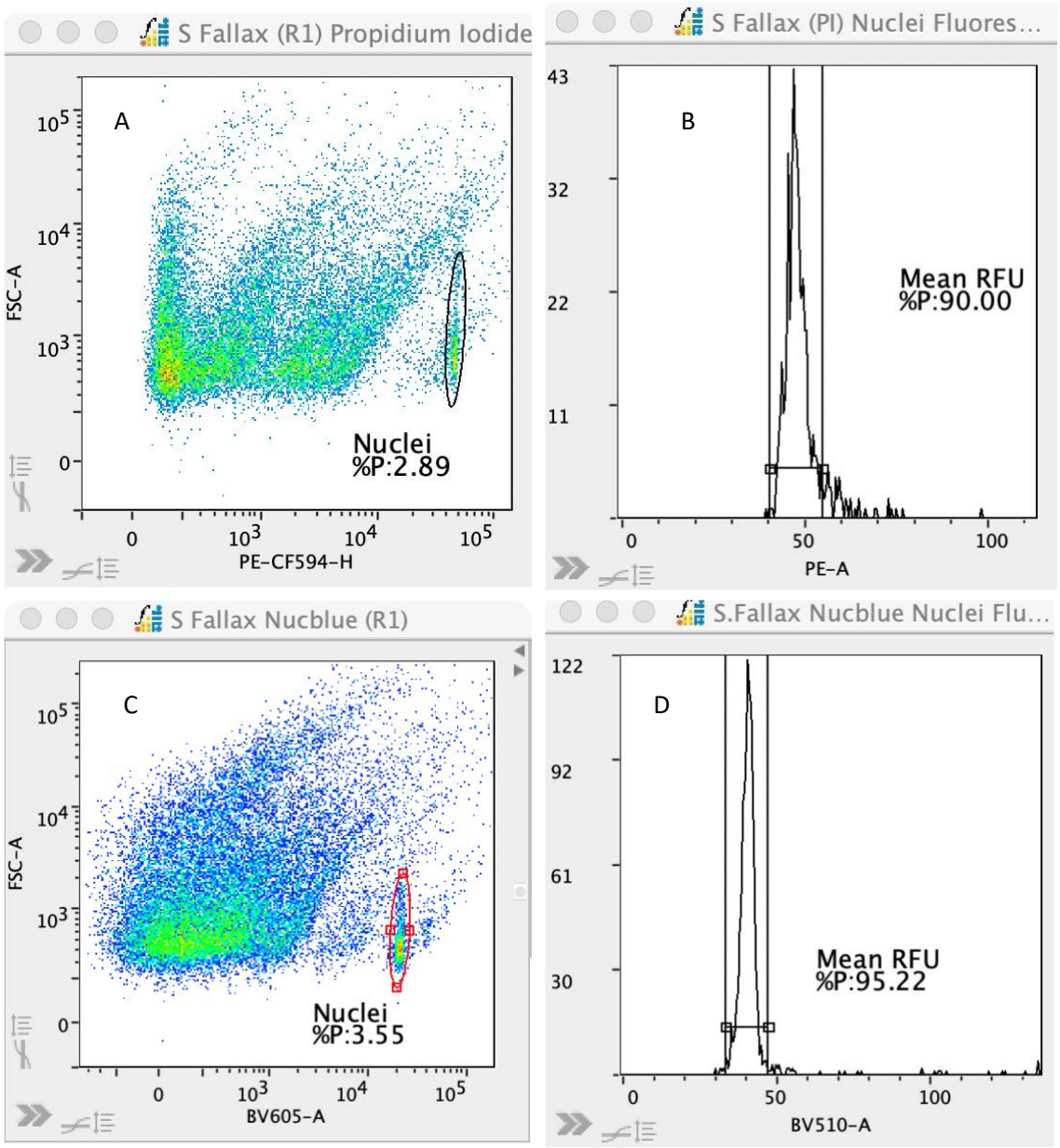


Figure 6 Acquisition and Histogram plots of S. fallax stained with fluorochromes PI (A-B) and Nucblue (C-D). Acquisitional dot plots plotted with channel volume fluorescence on the x axis and forward scatter (FSC-A, cell size) on the Y axis. Nuclei population percentage of the whole sample is represented as (Nuclei %P). Histogram plots are channel fluorescence (x axis) plotted against the count of nuclei (Y-axis). Mean RFU represents the proportion of nuclei in the polygon gate represented in the peak.

Acquisitional scattergrams for both fluorochromes outlined clear separations of nuclei populations from debris in diploid *S. palustre* (A-C). Fluorochromes of both kinds had a similar percentage of nuclei composition of the entire sample (PI-%P1.18, NucBlue-%P1.38). The histogram peaks (B-D) yielded similar numbers of nuclei (PI -15 events, NucBlue- 17 events). Histogram peaks in *S. palustre* displayed much broader peaks than in *S. fallax,* this was also apparent in terms of nuclei yield. Both fluorochromes had a greater success in binding with *S. fallax* than *S. palustre.*

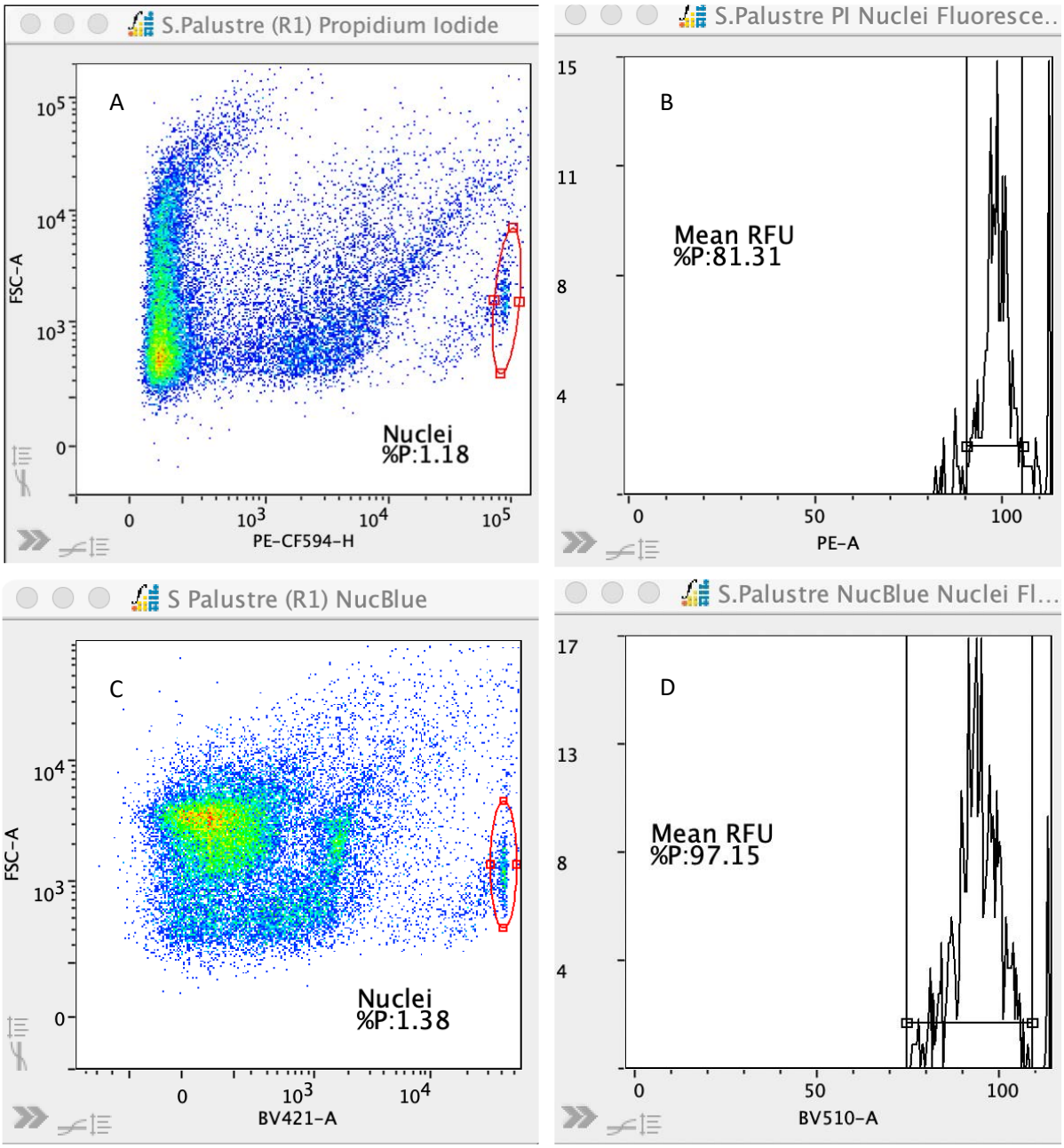


Figure 7 Acquisition and Histogram plots of S. palustre stained with fluorochromes PI (A-B) and Nucblue (C-D). Acquisitional dot plots plotted with channel volume fluorescence on the x axis and forward scatter (FSC-A, cell size) on the Y axis. Nuclei population percentage of the whole sample is represented as (Nuclei %P). Histogram plots are channel fluorescence (x axis) plotted against the count of nuclei (Y-axis). Mean RFU represents the proportion of nuclei in the polygon gate represented in the peak.

Discussion

Study specific method development has been strongly encouraged throughout plant cell flowcytometry (Dolezel et al., 2007, Galbraith, 2021). Presently genome estimation size and ploidy analysis is a common practice in plant material (Bainard and Newmaster, 2010) however preliminary testing is not always made apparent in academic literature, particularly in bryophyte material and even more so in *Sphagnum* based literature. This trialing offers further insight into how to optimize parameters when analysing nuclei in *Sphagnum* and highlights the importance of taking preliminary testing before conducting flowcytometry research to obtain accurate ploidy and genome sizes.

The primary hypothesis speculated that carrying out a range of trials that tested parameters involved in isolating and analysing *Sphagnum* nuclei would successfully refine methodology specifically for *Sphagnum* when using Flowcytometry. That hypothesis can be assumed to be true as many of the variables analysed provided substantial differences within each trial with all having an optimal.

In the first trial undertaken investigating the weight of material required for each sample, it was anticipated that a threshold would be identified in which an analysable number of nuclei would be apparent. The ideal sample weight would provide a clear population of nuclei unsaturated by debris remnant. When tested on a haploid sample (*S. fallax, Table.2)* clear differences in estimated genome sizes where apparent, with up to 43% difference between samples of different weights (20 mg-80 mg) genome results. The sample analysed with 40mg of material displayed a 95.45% similarity to the reference standard (40 mg – 0.42 pg Reference- 0.44 pg) and was the only sample that provided an RSE value lower than 0.05. Similar findings were apparent in the in the diploid *(S. palustre Table.2)* although the 40 mg sample did not hold the most similar genome size estimation of all samples analysed (40 mg- 92%, 80 mg- 94%) it was the only sample to have an RSE value less than 0.1.

It was clear to suggest that 40 mg of sample material was the optimum weight for analysis of both haploid and diploid *Sphagnum* specimens. Its visually apparent that 40 mg samples displayed more intact nuclei and a greater level of fluorescence (Fig 3, Fig 4). Samples of lower and higher weights displayed large variability. Across all samples Coefficient variation (%) did not differ drastically with most samples varying by 2-3%. This would implicate that the RSE values are high due to a low presence of nuclei not high peak fluorescence.

Reasons for unsuccessful nuclei extraction in terms of the larger material weights could be due to the nuclei adhering to mucilaginous compounds when being filtered as there is a greater volume of secondary metabolites (Dolezel et al., 2007). A greater volume of polysaccharides is released from cell walls due to a larger volume of material (Hajek et al., 2010) which increases the viscosity of the nuclear suspension resulting in a less successful filtration process and a lower reuptake of nuclear homogenate (Cires et al., 2011).

Unsuccessful yielding of nuclei in samples used with lower amounts of sample material (20mg) could be consequence of *Sphagnum*'*s* small genome size (Voglmayr, 2000, Dolezel, 2007). Other papers such as (Bainard et al., 2010) call for approximately 10mg of sample material however this undertook a wide screening of moss species looking at endopolyploidy not in *Sphagnum* specifically. This would imply that each individual species should be trialed to find optimum weight as appose to a general weight for all species being analysed.

Buffer choice within the study had a substantial difference in estimated genome size (29%). It was clear that the LB01 and Galbraith buffer proved to extract nuclei more successfully than that of the Tris buffer. This was evident as Tris provided the poorest histograms across all species analysed, with the lowest nuclei counts, broadest population peaks resulting in the highest RSE values. Tris provided a nuclei population of 0.45% in the total sample in contrast to the other buffers offering over 5 times the amount (2%>) of the total population.

Tris has often been seen to be relatively ineffective when trialed with other buffers (Bainard and Newmaster, 2010 Lourieo 2007). This could be due to the Tris buffer containing higher quantities of non-ionic detergents such as Triton-X100 which are effective at suppressing mucilaginous compounds when using higher quantities of sample material or species with bigger genome sizes (Lourieo 2007). Adversely concentrations that are too high can create a rapid denature of proteins and can prove fatal to nuclei as the clean-up process that detergents provide can become to rigorous (Brown and Audet, 2008).

The LB01 buffer provided the best results in both the haploid *S. fallax* and the standard material *P. sativum.* Although Galbraith provided the closest estimated genome size value in diploid *S. palustre,* LB01 buffer provided the most consistent values across all species analysed and provided consistently low RSE values. The addition of B-mercaptoethanol in LB01 could be the reason for it being the most successful buffer. The addition allows for the combat of cytosolic compounds found in bryophytes such as aromatics and terpenoids (Comisso et al., 2021) and more specifically acid like and flavonoid glycoside compounds found in *Sphagnum* (Fudyma et al., 2019). The use of B-mercaptoethanol reduces the sticking of debris to nuclei inhibiting a 'debris coating' which in turn stops the decrease in resolution of DNA when analysed (Nath et al., 2019).

The testing of a secondary fluorochrome was incorporated in the trials to try and streamline the process of sampling time. Nucblue is a fluorochrome that binds to A-T rich regions of DNA in contrast to Propidium Iodide which binds to all nucleobases. Nucblue would allow to remove the step of incubation before analysis and remove the need to add Rnase (a).

Samples stained with Nucblue did not display consistency between replicates (Table 3) in both the haplod *S. fallax* and the diploid *S. palustre.* RSE values where high when compared to the RSE values of Propidium Iodide. Samples stained with Nucblue did however comprise a greater nuclei percentage in the total population percentage. Nuclei counts were also higher in the samples stained with the Nucblue fluorochrome.

The unsuccess using Nucblue could be accredited to a lack of experience in acquiring nuclei using a new flow cytometer channel volume alongside the lack of application to bryophytes in literature. Nucblue is a variant of the Hoecht 3342 (Thermofisher.com) and fluoresces in a blue channel unlike the orange/red channel of propidium iodide. Factors such as autofluorescence must be considered when analysing in a blue channel as the green autofluorescence of plant cells emit light very closely to blue on the light spectrum (Bucevičius et al., 2018,). The potential of Nucblue is still to be considered, with more revision and incorporation of autofluorescence strategy's such as compensation could make the fluorochrome dye useful in high throughput screenings in future research. Nucblue is predominantly used on live cell cultures (Mazzinni and Danova, 2017) which would be beneficial if cell cultures where wanting to be used again in further study.

Propidium Iodide (PI) provided successful binding with consistent Mean 1C values (estimated genome sizes) in both the haploid and diploid samples. The haploid and diploid samples showing 95%> similarity in estimated genome size when compared to the reference standard. *S. fallax* displayed a

lower mean RSE value (0.08) whilst S. *palustre* displayed a high average RSE value (0.29). The nuclei count was substantially lower in the diploid *S. palustre* than in the haploid *S. fallax* which appeared to be prevalent over most of the trials.

Specimens of Increased levels of ploidy such as diploidy have been identified to have proportionally increase cellular traits (Robinson, 2018) alongside a significantly increased level of cytosolic compounds such as polysaccharides when compared to lower ploidy levels (Pham et al., 2019). This would suggest a similar situation to using greater amounts of material resulting in a poor filtration due to increased viscosity of the nuclear suspension. A revised version of the LB01 buffer with an increased level of *B*-mercaptoethanol could potentially be better suited to diploid *Sphagnum* specimens and may result in a more nuclei rich suspension as potential cytosolic compounds are eradicated.

Limitations within this section of study where mostly statical issues due to wrongful acquisition of data. At the time of analysis, it wasn't understood that nuclei histograms needed to be presented in a linear format for comparison between samples. Revisions were made throughout the course of the next stages of analysis. If time hadn't been a constraint during the methodology development, it would have been beneficial to test both fluorochrome stain durations and fluorochrome quantity instead of refereeing from cited literature. This could have aided in finding a peak staining period and concentration that could have provided a stronger fluorescence of DNA resulting in higher nuclei yields.

Conclusion

The primary objective of this stage was to test variables identified in flowcytometry of bryophytes to develop a functional protocol to competently analyse *Sphagnum* ploidy. This has been successfully achieved by highlighting an optimal weight of sample material, an appropriate buffer, a suitable fluorochrome, and acquisitional gating strategy all of which have contributed to creating a protocol that allows for more precise population peaks and higher nuclei yields. The presentation of data could have been easier represented by using linear scales. Diploid specimens could have been better represented by using a higher concentration lysis buffer. Developing a competent methodology has allowed for precision when looking into specialist specimens and validifies how to investigate *Sphagnum* through flow cytometry.

Final Procedure

1.12 Preparing intact Suspensions of Nuclei

1.12.a Gently extract 40 mg of capitula material from *Sphagnum* specimen using metal forceps and a four-place balance*. (When working with haploid samples there will be a considerable increase in the amount of capitula's due to haploid being lighter than diploid specimens).*

1.12.b Place sample material in a petri dish. Be sure to mark-up petri dishes appropriately to avoid confusion later in analysis.

1.12.c Remove 25ml lB01 lysis buffer tubes from -20°C freezer and place in a 37°C incubation room for 20 minutes to bring up temperature to an ice-cold liquid.

1.12.d Remove PI, RNase (a), and razor blades from refrigerated storage, and put on ice using an ice box. *(Ice cold razor blades aid in providing a cleaner chopping procedure and remove risk of nuclei sticking to razor blade.)*

1.12.e Once buffer has reached a liquid temperature, pipette 1.2ml on top of the sample in the petri dish.

1.12.f Place the petri dish on a slant to allow the buffer and sample to occupy a smaller surface area. *(Slanting helps nuclei and liquid buffer from dissipating across the petri dish)*

1.12.g Chopping occurs immediately using an ice-cold razor blade for a short vigorous period (8 Seconds). *(It is important that the sample is chopped and not squeezed. Sphagnum material should be visually broken with remnants of chunks, the sample should not be macerated. It is vital to note that chopping is used as a support to ease nuclei, the buffer is the predominant driver of the process.)*

1.12.h Once chopping has been finalized pipette the homogenate thoroughly (4-5 times). *(Make sure that pipetting is done gently to avoid bubbles, or the suspension of nuclei will rapidly deplete.*

1.12.i Apply a sterile 0.42 µm PET nylon mesh filter on top of a suitable flow cytometer tube and pipette 1m l of the homogenate through the filter. *(Pipette carefully to avoid splash back which can result in a loss of nuclei suspension).*

1.12.j Store filtered suspensions in cold storage 4°C on ice for 10 minutes before applying 150 µg of DNA fluorochrome stock solution Propidium Iodide (PI) and 50 µg Rnase (a) simultaneously.

1.12.k Vortex samples to assist in the binding of PI and Rnase to DNA.

1.12.l Analyse immediately.

1.13 Preparation of Flow Cytometer

I) Firstly, turn on the flow cytometer (BD Celesta) and check the interface for any fluidics notifications. Possible situations include emptying the waste container and changing the sheath fluid container, situations such as these will glow red indicating that it needs to be acted upon.

II) Both the waste container and the sheath fluid can be found in the station below the cytometer. To remove or change press the STANDBY button on the interface and remove with caution wearing the appropriate PPE.

III) Press the restart button on the fluidics station and once the instrument has registered the change the light will change from red to green. The cytometer is now ready to be run.

IV) Now open BD Cell Quest Pro 2 software and run the calibration beads before every analysis. *(This makes sure that the lasers are calibrated correctly, giving a reliable reading.)*

1.14 Acquisition of Nuclei

V) Once calibration has finished successfully Introduce the vortexed sample to the instrument via the Sample Injection Port (SIP). *(This can be achieved by moving the sheath arm to the left and attaching to the port.)*

VI) Slide the sheath arm back into the central position and press RUN on the cytometers interface. (The hovering yellow light will move from standby to a green colour)

VII) The flow rate should always be set to LOW when acquiring nuclei to avoid exaggerated readings on the instrument. This also stops the instrument from taking up the nuclei suspension to quickly when trying to locate and gate nuclei.

VIII) Press the acquire in the inspector of cell quest (found on the left-hand side). The instrument will now start acquiring events.

IX) Adjust parameters to visualise all events. Set voltage for FSC to 407. SSC to 448. Make sure both boxes are checked to be represented in logarithmic scales.

X) Set up an acquisitional dot plot in Cell Quest contrasting FSC-A (Cell size X-axis) vs SSC-A (Cell complexity Y-axis).

XI) It will be immediately apparent that a population cannot be seen. This is due to an overlap of debris and nuclei due to cells being the same size.

XII) To separate the desired population from debris, a fluorescence channel needs to be selected as the x-axis and plotted against FSC-A (Cell size) on the Y axis.

XIII) Select the PE-CF59 channel and adjust the voltage to 526 making sure the logarithmic box is checked. The population of interest will fluoresce in the desired channel as fluorescence will emit within the channels light spectrum and be visibly separate from the debris as all the nuclei of one population should fluoresce in one section in the channel and can be seen represented as a vertical line.

XIV) A series of elliptical gating is needed to isolate the population from debris. Once the population of interest has been isolated, doublet discrimination is needed to factor out nuclei that could have potentially coagulated whilst been analysed. If not carried out doublets can give a false reading of higher ploidy as the instrument would present the data point as twice the fluorescence indicating twice the volume of genetic material.

XV) Create two scattergrams plotting SSC-A vs SSC-H and FSC-A and FSC-H. This plots area of cells against the height of the cells. Create a gate around the nuclei that are proportionally increasing, outliers are indications of coagulated nuclei.

XVI) Once gating is complete, analysis of nuclei can occur. Open the parameters and adjust the voltage of the PE channel to 599 and make sure it is in a linear scale.

XVII) Create a Histogram to represent the DNA peaks. This graph will be presented as PE on the x-axis and nuclei count on the Y axis.

XVIII) Adjust the voltage so the DNA peak (Haploid -Sample) is at a 50 value on the x axis. When analysing a diploid sample using the same parameters the DNA peak should fluoresce at the 100-value due to double the level of genetic material.

XIX) Now acquisition is complete change the total number of events to be run to 30,000 in the inspector.

XX) Run the instrument on MED flow collect 30,000 events and save the data.

Chapter Two

Screening of Ploidy and Polypoidal Inducement

Introduction

2.1 Polyploidy in Plants

Polyploidy is a genetic trait in plant species across the globe, these plants have all experienced a form of whole genome duplication during their evolutionally history (Pele et al., 2018). Plants that are regarded as polyploidal possess three or more homologous sets of chromosomes. (Corneillie et al., 2018), and occur in two types; autopolyploid, which are initiated from diploid multiplication and allopolyploids which are consequential of the hybridization of two haploid genomes (Barker et al., 2016). Polyploids develop a set of enhancements which increase the quality of biological functioning commonly known as Heterosis. Heterosis exhibits greater biomass, speed of development, and higher rates of fertility throughout the plant (Birchler et al., 2010) which have been critical in horticulture and agriculture for increasing plant yield (Sattler et al.,2016).

2.2 Ploidy and Polyploidy in the Sphagnum L genus

Ploidy levels in *Sphagnum* are naturally either haploid or diploid outlined in (Temsch, 1998) with 86% of species being haploid. Consequentially *Sphagnum* species have a particularly low DNA content, lower than that of most other plants. Even though *Sphagnum* is not a higher species it is apparent that it has unique physiological and morphological adaptations which help sequester carbon (Kostka, 2016). In terms of enhancing the outcomes of *Sphagnum* farming It would be of great importance to enhance its outreach and capabilities. Initiating a polypoidal state in *Sphagnum* is a step that could pose a range of potential advantages.

Polyploidization has been shown to induce more compact growing genotypes this is desirable as these genotypes tend to develop compact growing plants (Denaeghel et al., 2018) increasing the level of robustness. It has been outlined that the increased allele number in polyploid genes cover harmful recessive mutations (Gu et al., 2003), ensuring against the loss of contribution to the gene pool. This would prove extremely valuable in a farming scenario as it eliminates potentially massive risks to productivity. A second being the increasing flexibility to environmental change, gene duplication occurs in polyploidization which support the development of new gene function (Long and Langley, 1993) increasing phenotypic plasticity allowing *Sphagnum* to become a more domesticated plant. Lastly, the final enhancement would be heterosis. Increasing the size and growth rate in *sphagnum* cells could potentially increase biomass concentrations in *sphagnum* not only beneficial to offset carbon via the means of *Sphagnum* farming but also for peatland restoration (Pouliot et al., 2015). Polypoidal heterosis initiates stress resistance and drought tolerance (Van Laere et al., 2010) this is due to the increase of flexibly in the genome (Levin 2002). Research into inducing polyploidy is poorly represented in the relevant literature which would suggest why this research should be undertaken.

2.3 Wide Screening of Material

Beadamoss Micropropagation Services are in possession of a large range of *Sphagnum* species, of both believed haploid and diploid nature. Carrying out a large screening firstly allows for a better understanding of ploidy levels within their archives and secondly widens the possibility of finding individuals with higher levels of ploidy. Understanding different ploidy levels in different species will aid in creating suitable matrix of species for *Sphagnum* farming. Wide screenings consist of large amounts of samples and species (Koleva et al., 2002, Bainard, 2010) and generally require protocols that can run high throughputs of samples efficiently without comprising quality of results.

2.4 The function of oryzalin

Oryzalin is a herbicide that belongs to a group of dinitroanilines (Wacker et al., 1988). The herbicide is synthetic and is often used as weed control (Ascough et al., 2008). Oryzalin has an affinity to create polyploidy in plants. Oryzalin is not the only chemical that creates such possibilities, chemicals such as Colchine are also widely used to create individuals with increased levels of ploidy. Recently oryzalin has been the favorable choice firstly due to oryzalin being used in micromolar quantities instead of millimolar like Colchine, and its widely regarded as being a less toxic product to use (Wannakrairoj & Tefera, 2003). When oryzalin and Colchine have been used in the same study oryzalin often provides a higher number of tetraploids (Ascough et al., 2008) provide more stable ploidy induction and a higher rate of survival in explants (Zlesak et al., Pliankong et al., 2017).

Oryzalin works by interfering with the cytoskeleton of nuclei and begins the process of depolymerisation of microtubules preventing meiotic spindle formation (Caperta et al., 2006), this essentially allows the duplication of genetic material to occur within the cell, binding to plant tubulin (Morejohn, 1987) inhibiting cell division resulting in cells with double the number of chromosomes. If this happens at a sufficient level in the cell cycle, then polyploid individuals can result.

2.5 How Rare is Polyploidy

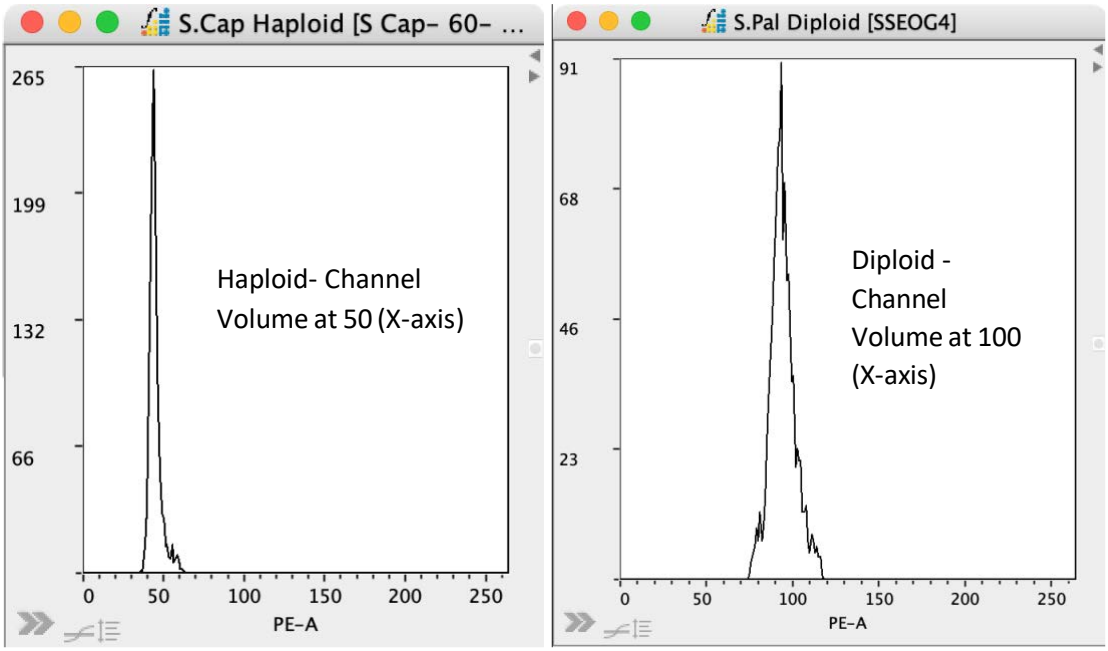
Polyploidy is found to be extremely rare within mammalian cells, however it is a much more common genetic process in plants (Svartman and Stanyon 2005). Plants from various taxa have varying levels of polyploidy. In its highest frequency, angiosperms have been identified as 1 in every 100,000 individuals (Woodhouse et al.,2009). In bryophytes a substantially smaller history of (5-10%) of mosses having polyploidy in their lineage (Sastad 2004) . Unfortunately, the *Sphagnum* genus is not one of the mosses that have displayed natural occurrences of polyploidy directly from the same set of chromosomes (Bainard et al., 2010). *Sphagnum* in the southern hemisphere has noticeably gone through hybridization to create the allopolyploid *S. falcatulum* which spans a considerable portion of the Holantartic (Karlin and Smouse 2017). In the northern hemisphere smaller dispersals of a more localised nature have produced polypoidal *S. trondelagicum* In Norway (Stenoien et al., 2011). Although these polyploids are apparent not all new polyploidizations will lead to substantial species diversification over the long term. (Arrigo and Baker 2012). To date allopolyploid hybridization has not featured in UK *Sphagnum* communities, possibly due to these hybridizations forming from preglacial ancient lineages in polarized places (Stenoien 2010, Karlin and Smouse, 2017).

Inducing polyploidy (artificial auto -polyploidy) has seen to be successful in a whole range of plant species and wouldn't be considered a rare occurrence. The prominent success of polypoidal inducement has been seen in angiosperms (Juliao et al., 2020, Mo et al., 2020) and in grafted crops (Ruiz et al., 2020). Succusses of polypoidal inducement in Bryophytes is questionable as no currently published study has been undertaken trying to increase levels of ploidy in bryophytes more specifically in the *Sphagnum* genus.

2.6 Measuring Fluorescence and its Application to Ploidy and DNA Content

Flow cytometry functions on passing single cells through a chamber 1um thick. These cells pass through a series of lasers which respond to a fluorochrome dye attached to the cell DNA. The dye used is excited at a certain wavelength and emits light at a wavelength further down the light spectrum. Each individual event is recorded, and the complexity of the DNA appears at a certain point along a channel in which the fluorochrome emits. Cells with higher amounts of DNA are found further down the fluorescence channel as the laser is receiving more response to the fluorochrome as there is more DNA to bind to.

DNA content and Ploidy values are extracted from the mean channel peaks as seen below. Channel volume dictates what level of ploidy the specimen is. Channel volume (X-axis) indicate two volumes the first being a haploid value (50) and the second value being diploid (100) these volumes are where haploid and diploid specimens are supposed to appear. A mean channel volume can be extracted from the sample and used in an equation to create estimated DNA values (fig.2). Using flowcytometry and fluorescence is one of the most versatile methods of measuring ploidy it has functional applications to DNA content with presentable formats (Dolezel 2007).



The overarching aim of this chapter is to investigate levels of ploidy in a select group of micropropagated *Sphagnum* species, conducting a wide screening of specimens both subject to inducement and not.

The objectives of this chapter are:

- Quantify levels of DNA (pg) in Specimens provided by BeadaMoss through a wide screening of material.
- Categorize species from various batches into haploid and diploid to develop an ongoing archive.
- Assess changes in relative fluorescence in specimens induced with Oryzalin.

It was originally hypothesised that specimens of a haploid nature would provide better responses to the lower concentration of 60 μm, and the specimens thought to be diploid would be more responsive to the 120 μm concentrations.

Methods

2.7 Inoculation of Oryzalin

To gain a comprehensive understanding of oryzalin's effect on *Sphagnum* multiple species across the genus were considered for analysis. To achieve this, the inoculation process was carried out externally at BeadaMoss research laboratory. Due to oryzalin being an herbicide there was an expectation that over exposure could lead to destruction of plant nuclei, alternatively an under exposure could result in no development of ploidy increase. It was agreed that two thresholds of oryzalin exposure would be investigated. Refence exposure rates of oryzalin in bryophytes are not apparent when examining the literature. 120 μm (μmol/L) at 24hr has been seen to be the most prominent in creating tetraploids (polyploidy) In non-bryophyte material (Ascough et al., 2008), and this was chosen as the higher standard. Due to *sphagnum*'s small amounts of genetic material an exposure of half this (60 μm) was used in parallel.

2.8 Sphagnum Coding and Batching of Sample Material.

The codes that are applied to *sphagnum* species are presented throughout the chapters of research and are representational of region of collection and individual isolate used. The codes are not an essential point to this chapter of research and are apparent to distinguish different individuals that have been analysed from a specific species only.

Achieving a high throughput screening of oryzalin induced *Sphagnum* specimens was a primary objective. To ascertain such a screening many specimens would need to be processed. Limited space within growth chambers and the need for juvenile material made it difficult to hold all the samples at the university. For this reason, an agreement to use a batch delivery schedule. The use of a plan also allowed for a logical structure of progression as more became understood about oryzalin in *Sphagnum.*

The oryzalin study consisted of three individual batches. Batch one provided a comparison of both the lower concentration of oryzalin and the higher concentrations of oryzalin (60 μm and 120 μm). Batch two was entirely the higher concentration as it proved to show more variance than the lower concentration. It consisted of both thought to be haploid and diploid species.

Sample Codes-

The sample codes refer predominantly to internal referencing markers and differentiating species by the regionality of sampling.

Batch Structure

* Each species came with several jars of standard material provided by micropropagation services to create a comparative non inoculated control. (Controls consisted of four jars per code of which three replicates were taken from each totaling 12 replicates per code.)

* Batches of inoculated material came with appropriate coding and 10 jars (clones) assigned to each batch code.

Batch 1 – Initial undertaking of inoculated material. This batch is set to investigate the, reactive differences between longer exposures to oryzalin versus shorter exposures, how this differentiates in relative fluorescence and if these exposures propose alteraions/ duplications in genome sizes.

- 60 μm samples- Two known haploid species *S. fallax* SFLG3 and *S. capillifolium* SAWBT, and two diploid species, *S. palustre* SSFIE and *S .papillosum* SPY27.
- 120 μm samples- Two known haploid species *S. fallax* SFLG3 and *S. capillifolium* SAWBT, and two diploid species, *S. palustre* SSFIE and *S. papillosum* SPY27.

Batch 2 – Second batch aims to expand from finding optimum exposure threshold and apply this to a greater number of species in the *Sphagnum* genus. Test the reactive differences between haploid species, diploid species, and contrast both.

- 120 μm- Haploid- *S. fallax (*SFLHE), *S. squarrosum* (SQGWB), *S. denticulatum* (SDSRM),
- 120 μm- Diploid- *S. russowii* (SWGNI), *S.majus*(SJGEC)

Batch 3 – Final addition of regional variants.

- 120 μm- Haploid- *S. fimbratium* (SIGHA)
- 120 μm-Diploid – *S. palustre* (SSEOG), *S. palustre (SSBEC), S. palustre (SSGUS)*

Practical Methods

2.9 Extraction

Sample material was analysed following the procedure refined in chapter 1. *Sphagnum* material was lightly chopped in 1 ml of LB01 lysis buffer in a titled petri dish. (Oryzalin was added to the plaiting media externally prior to this study by BeadaMoss). The suspension was then homogenised via pipetting three times whilst actively avoiding the creation of bubbles. One final 1 ml of the liquid suspension was then passed through a 0.42 μm nylon mesh filter into a flow tube. The sample was then incubated on ice for 10 minutes. Rnase (a) added at 0.50 ug and Propidium Iodide (PI) added at 150 ug were incorporated into the sample simultaneously before flowing cytometric analysis. (See Final procedure – reference number).

2.10 Acquisition and Post Analysis

Upon readying samples for analysis, initial procedure checks were carried out the same as (Chapter 1- preparation of flow cytometer). Samples where analysed for levels of fluorescence using a BD FACs Celesta. Haploid peaks where set to a channel volume of 50 and recordings occurred up to 30,000 events on the medium flow setting. Data recorded included fluorescence histogram peaks including the mean, median, and Coefficient of variance. Acquisition of nuclei was achieved using the procedure outlined in Chapter 1 (reference number). Once an entire data set had been attained from the flow instrument, data was further analysed using Flow Logic to interpret mean fluorescence values and aid in forming normalised data.

2.11 Data Representation

Data was presented predominantly in normalised bar charts. As the histogram peaks provided rather broad peaks in some cases this was the most interpretable means of visualising differences between inoculated samples and the controls. This was presented as a control displaying the value of (1) for its 1C value.

Objective methods

2.12 Measuring using Estimated DNA Amounts and Ploidy Levels

As this study is centered around understanding ploidy levels representation of ploidy has been included throughout although due to the potential level of mixoploidy that oryzalin can create (Ascough et al., 2008) estimated DNA amounts provide a numerical metric alongside the level of ploidy. Referring to C-values, ploidy values alone can be unrevealing at times and don't reflect the level of variance particularly in specimens that haven't gone through an entire chromosome duplication. For this reason, data throughout this chapter has both metrics.

2.13 Testing Micropropagated Sphagnum Verses a Reference Standard

Before being able to test oryzalin induced species for polyploidy, standard non inoculated material grown in the same format was to be tested. Firstly, this would provide BeadaMoss with an understanding of the ploidy levels of their already existing cultures and secondly allow for a direct comparison between micro propagated material and Kew reference standards. This would also provide a competent standard in which the only variable between the inoculated sample and the standard would be the incorporation of oryzalin.

2.14 Measuring Haploids and Diploids

Haploid samples were analysed using normalized bar charts and compared against a non-inoculated standard (referred to as 1). Diploid samples were analysed using the same procedure.

2.15 Contrasting Performances of Concentrations of Oryzalin

To further refine the screening process samples of 60 μm and 120 μm where to be analyzed against each other in comparison to a non-inoculated control. Samples where all grown in the same conditions under the same parameters. It was thought that by trialing simultaneously a clear optimum concentration would be found for individual species.

2.16 Measuring Induced Samples Verses, the non-inoculated standard.

Measuring against the standard in a normalised bar graph format allowed for a direct comparison to how the samples had reacted to the oryzalin and if they display a higher fluorescence intensity when compared to the non-inoculated material. A higher fluorescence intensity is indicated by taller bar plots and suggest that there is a higher amount of genetic material in the sample.

2.17 Measuring Performance Between Species with Multiple Codes

Throughout the samples received from BeadaMoss some species came with sets of more than one code. This highlighted the difference in how different species from different origins reacted with the oryzalin treatment. The species present for this analysis consisted of *S. fallax* having two individual codes (SFLHE, SFGC3) and *S. palustre* (SSFIE, SSEOG, SSGUS). This would provide data on relative fluorescence intensity, and genome size estimations within species response in both a haploid and a diploid example.

2.18 Samples of Interest

Samples of interest are samples that displayed more than a 30% increase in fluorescence. Due to many samples showing an increase of more than 20% it was thought that using a 30% threshold would provide a suitable number of samples to further analyse. Samples that displayed this level of interest where further analysed in FlowLogic using comparative peak analysis to determine quality of results.

Results

2.19 Standard Material

Table 4 Highlights standard material used as a control incongruence with oryzalin induced material. Individuals in species are represented by species code. Estimated DNA amount (1C value) are provided from flowcytometry analysis and is measured in picograms (pg). Primary DNA amounts are contrasted against a reference estimated DNA amount and are represented in picograms also. Ploidy level is also provided to distinguish between haploid and diploid species. Four replicates where taken per species code.

Species	**Species Code**	**Reference Estimated DNA amount 1C (picograms**	**Estimated DNA Amount 1C (Picograms)**	**Ploidy Level**
S. fallax	SFG3	0.44 (pg)	0.44 (pg)	Haploid
	SFLHE		0.49 (pg)	Haploid
S. capillifolium	SAWBT	0.46 (pg)	0.46 (pg)	Haploid
S. squarrosum	SQGB	0.46 (pg)	0.44 (pg)	Haploid
S. fimbriatum	SIGHA	0.46 (pg)	0.43 (pg)	Haploid
S. denticulatum	SDSRM	0.39 (pg)	0.61 (pg)	**?**
S. majus	SJGEC	0.81 (pg)	0.79 (pg)	Diploid
S. papillosum	SPY27	0.93 (pg)	0.90 (pg)	Diploid
S. russowii	SWGNI	0.93 (pg)	0.91 (pg)	Diploid
S. palustre	SSFIE	0.92 (pg)	0.91 (pg)	Diploid
	SSEOG		0.92 (pg)	Diploid
	SSEBC		0.89 (pg)	Diploid
	SSGUS		0.95 (pg)	Diploid

(Table.4) contrasts estimated DNA amount and ploidy level of standard micropropagated material grown at BeadaMoss against a reference standard provided by Kew. This was used to guide results that came from oryzalin induced species.

All species analysed provided suitable standards within (0.05 pg) 1C values when compared to the reference standard. This would suggest that the acquisition of data is correct, and the suspected nuclei populations are in fact the nuclei. Model standards for investigating the inoculated material.

The only sample that did not provide a suitable 1C value was *S. denticulatum* which was neither close to the 1c value (0.39 pg) or 2C value (0.78 pg) which could be a potentially different species.

2.20 Induced Material 60 µm vs 120 µm oryzalin concentrations

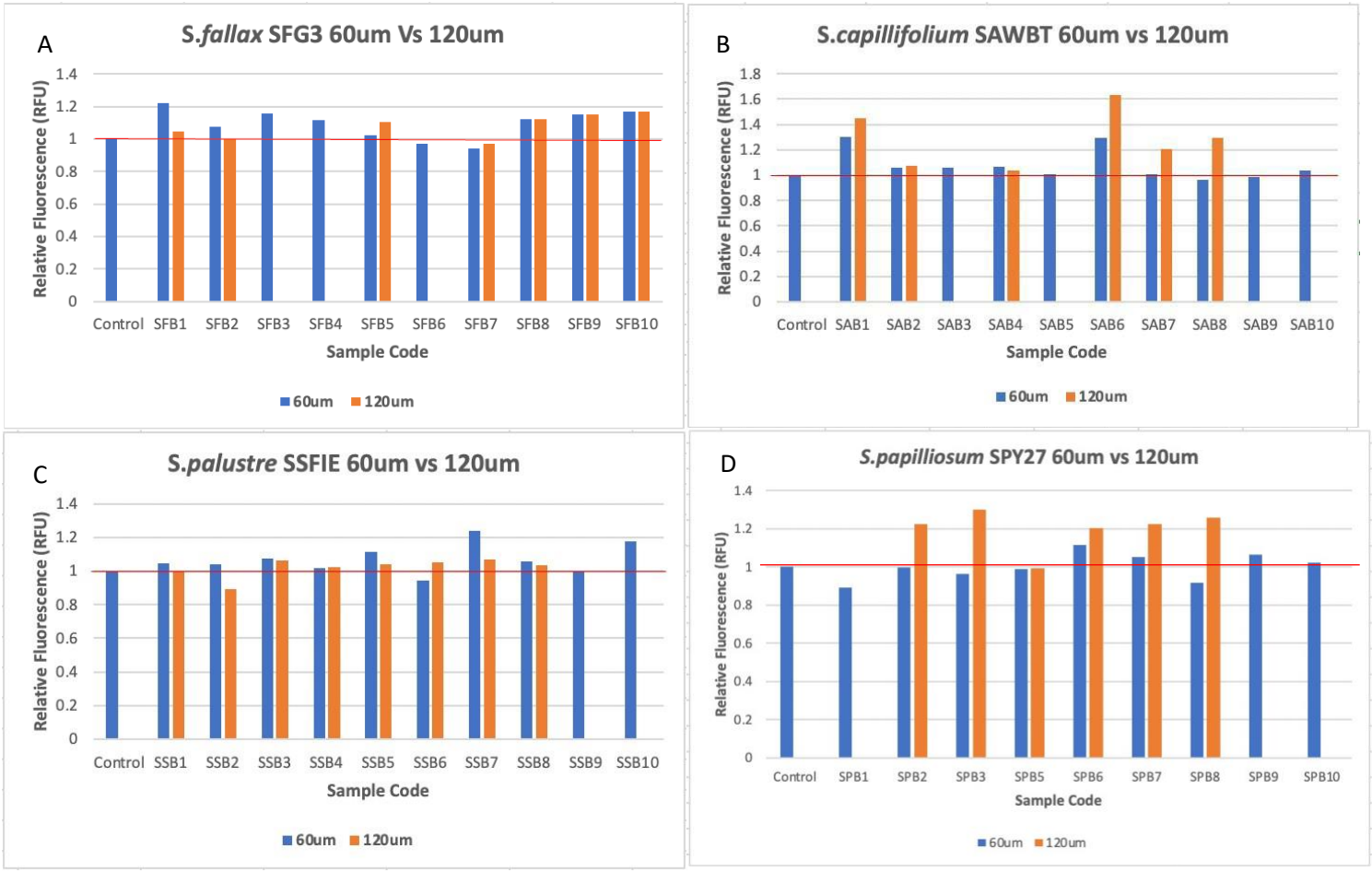


Figure 8 Displays normalised bar graphs of oryzalin induced species at two different concentration rates. The first being (60 µm) represented by the blue bar and (120 µm) represented by the orange bar. Samples are measured against a normalised control valued at 1. Samples can be found on the X-axis via the sample code. On the Y-axis relative fluorescence (RFU).

Oryzalin treatments displayed a variety of fluorescence between the lower inoculation of (60 µm) and the higher concentration of (120 µm). In the haploid *S. fallax* (A) both concentrations provided similar levels of fluorescence. The 60 µm concentration displayed one of the highest fluorescence increases (20%). Only two samples had a lesser level of fluorescence than that of the control. 60 µm concentration displayed a 9% average increase across all samples, 120 µm provided a 7% average increase.

In the haploid *S. capillifolium* (B) the 120 µm treatment displayed a greater increase in fluorescence than the 60 µm treatment. Select samples (SAB1 SAB6) had increases between 40% and 60%. An increase was also seen in select samples in the lower treatment (60 µm) but at lesser increase of 25%. Samples treated with the lower concentration of oryzalin had fluorescence values closer to the fluorescence value of the control, with some samples providing a lower level of fluorescence than the control. 60 µm concentration had an average increase of 7% across all samples whilst 120 µm provided a 27% increase.

In the diploid *S. palustre* (SSFIE) (C) both treatments provided relatively low levels of increased fluorescence when compared to the performance of the haploid samples. The lower treatment of oryzalin appeared to outperform the higher concentration marginally with one sample having an increase of fluorescence by (22%). The 60 μm concentration had an increase of 7% on average whilst the 120 μm concentration displayed an average of 2% across all samples.

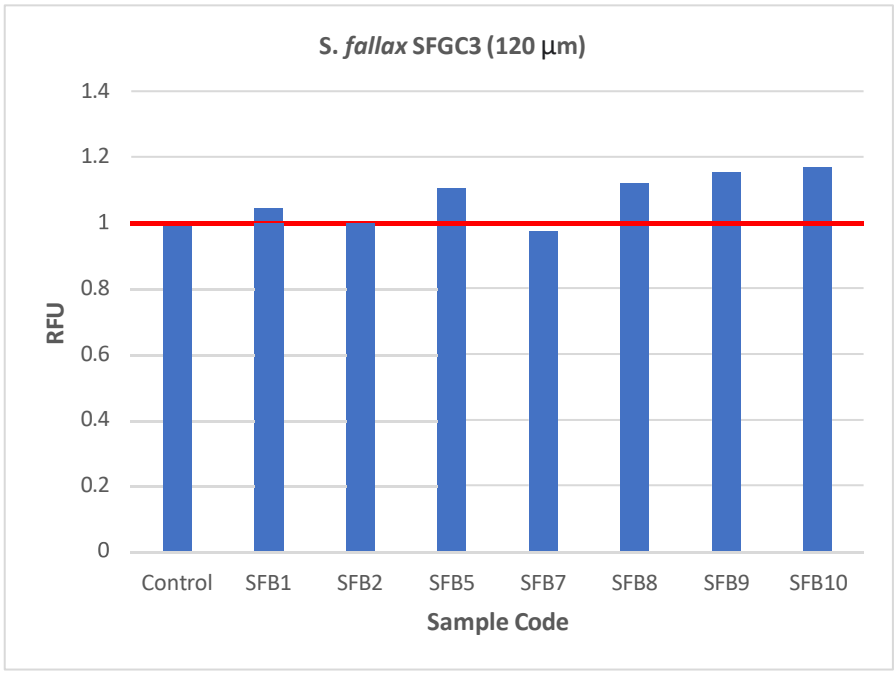
In the diploid *S. papillosum* (SPY27) (D) the higher concentration treatment provided greater levels of fluorescence consistently throughout most of the analysed material. Samples analysed with the lower treatment dose displayed a similar level of fluorescence to that of the control. SPB1 provided a fluorescence value 15% lower than the control. The 60 μm treatment provided 0.1% average increase, whilst 120 μm treatment provided an average increase of 20%.

Both treatment types displayed samples with fluorescence values lower than the control which indicates that oryzalin can initiate effects that are averse to the expectation of solely increasing ploidy levels in *Sphagnum* material.

Although the lower treatment batch outperformed the higher treatment batch in two of the species analysed it appeared that the 120 μm treatment provided a wider array of increased relative fluorescence when compared to the control. It was therefore decided as the optimal treatment out of the two concentrations. Samples inoculated with 120 μm Oryzalin where then pursued for further analysis.

2.21 Haploid 120 µm Species Analysis

S. fallax



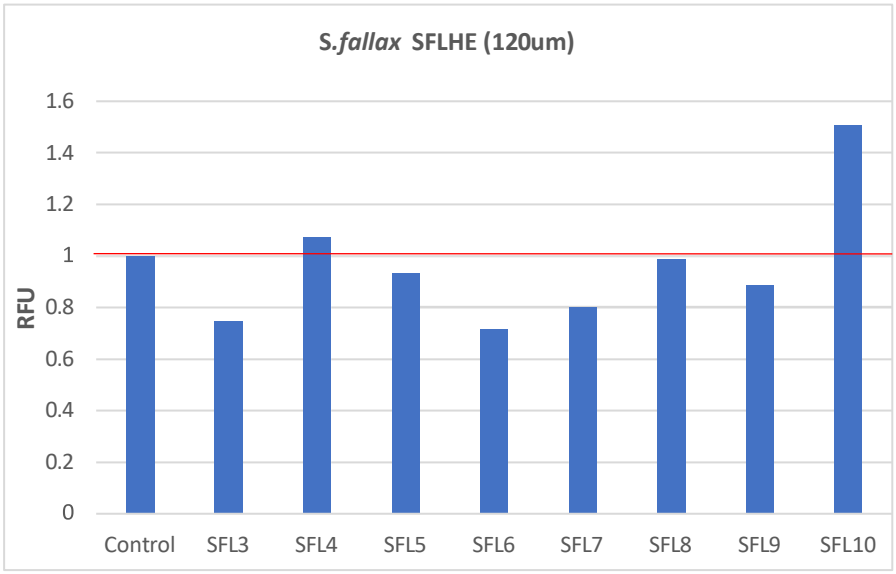


Figure 9 S. fallax normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration. Bar charts are both specimens from the *S. fallax* species labelled with different codes due to location of origin.

60µm oryzalin inoculated S. *fallax* (SFG3) displayed very little relative fluorescence increase when compared to the control l with an average of an 8% increase in fluorescence. A significant increase was not apparent in any of the samples analysed. Alternatively, The SFLHE batch of *S. fallax* appeared to be more response to the oryzalin treatment in means of both increasing and decreasing relative fluorescence to the oryzalin treatment. One replicate displaying a 50% increase in fluorescence. Average fluorescence across the inoculated samples was 5% less than that of the control.

S. capillifolium

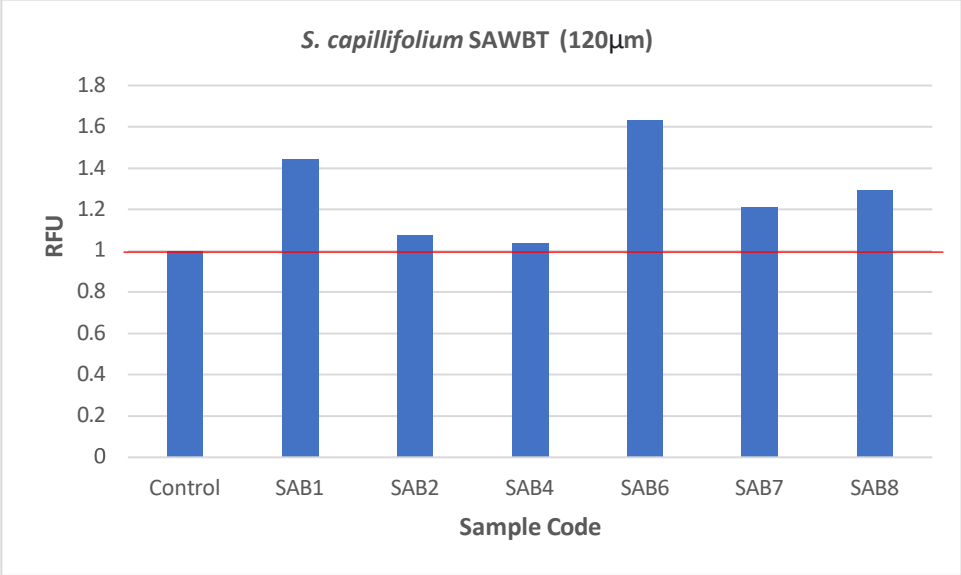


Figure 10 S. capillifolium normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration.

S. capillifolium provided a large amount of increased variance with two samples having an increase of (44% -SAB1) and (63% -SAB6). All samples displayed an increase of fluorescence when compared to the control suggesting a positive effect to the treatment. Samples displayed an average increase in fluorescence by 28%.

S. squarrosum

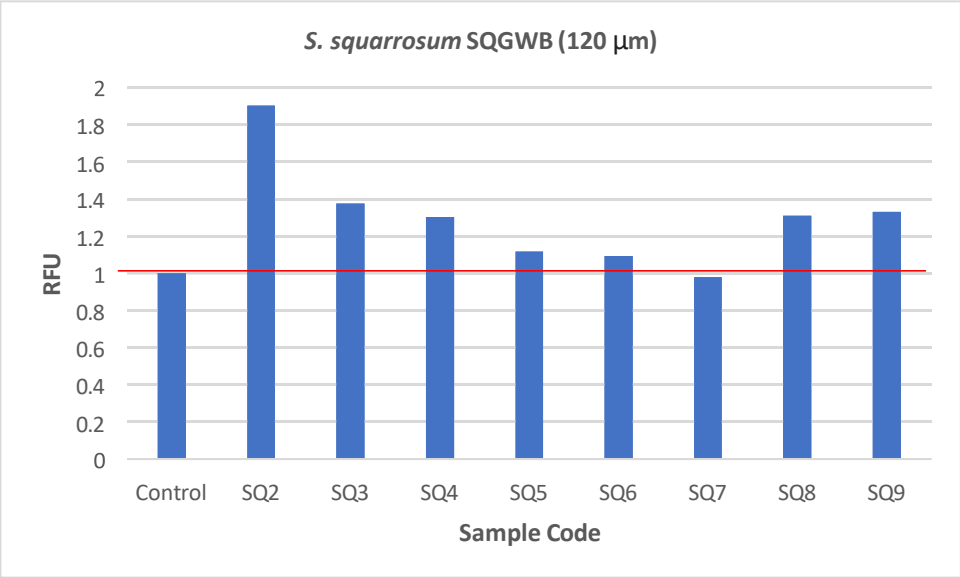


Figure 11 S. squarrosum normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm *concentration.*

S. squarrosum appeared responsive to inducement with several replicates showing large increases in relative fluorescence at 120 µm treatment. Samples, SQ3, SQ8 and SQ9 all displayed a 30% increase in fluorescence when compared to the control. Sample SQ2 had an increase of 90% which would indicate that all nuclei have almost entirely increased level of ploidy. Average increase of fluorescence was 30% across samples.

S. fimbriatum

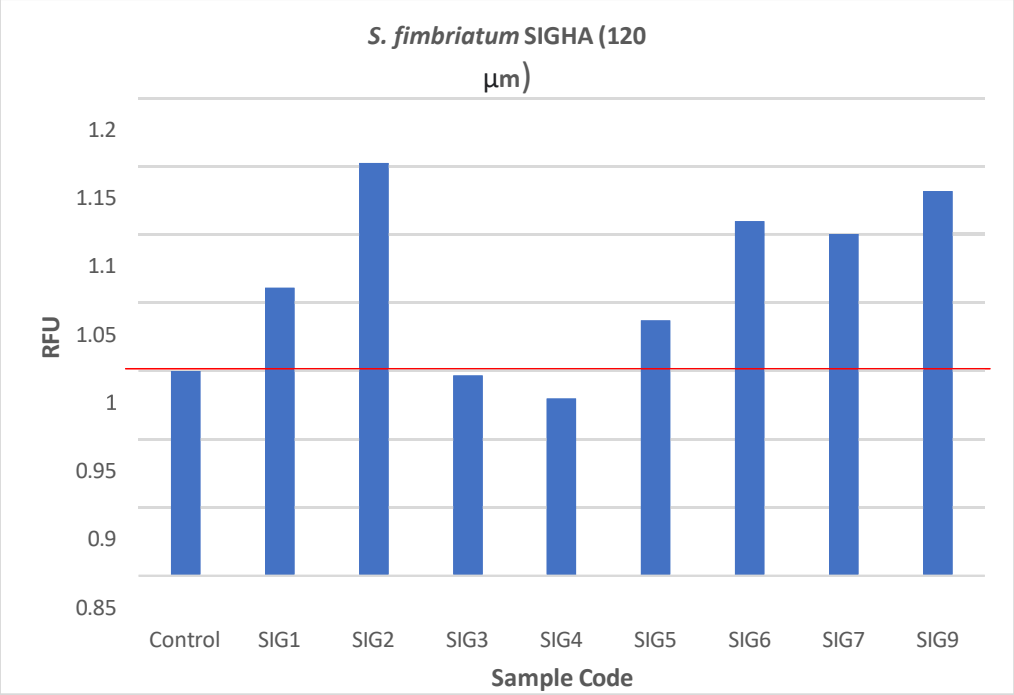


Figure 12 S. fimbriatum normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration.

S. fimbriatum did not provide any individuals with a substantial increase in fluorescence. SIG2 provided the highest increase at 15% whilst SIG4 provided a result 2% lower than the control. Average fluorescence was 7% higher than the control across the samples analysed.

S. denticulatum

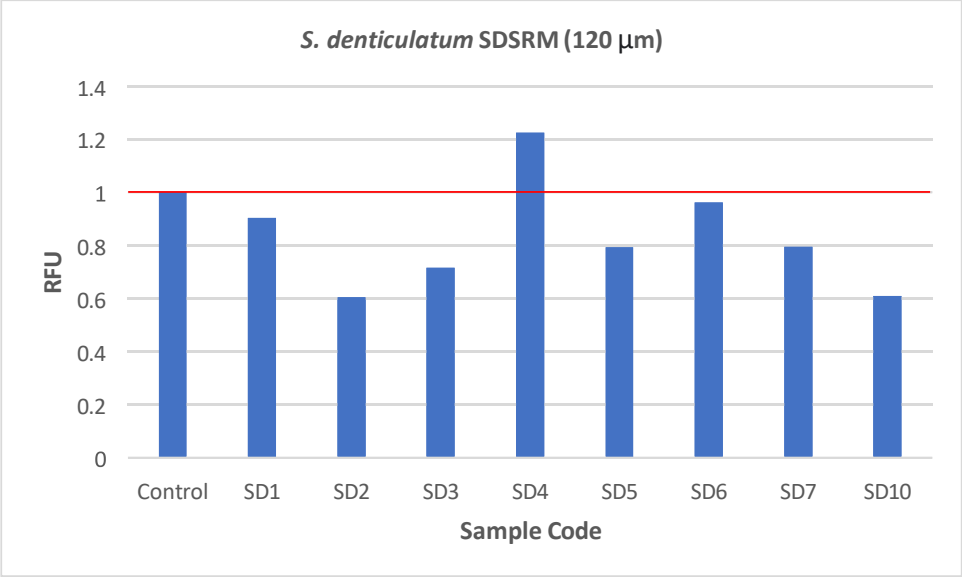


Figure 13 S. denticulatum normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration.

S. denticulatum had an adverse response to the oryzalin treatment. Only one samples (SD4) displayed a positive reaction to the inoculation having a 22% increase in level of fluorescence. The other samples analysed provided substantially lower levels of fluorescence with SD2 displaying 40% less fluorescence than the control. Average fluorescence across the samples was 18% less than the control. *S. denticulatum* was the only species that didn't provide an accurate reference standard which could be reflective in the accuracy and performance of inoculated specimens.

2.22 Diploid 120 µm Species Analysis

S. majus

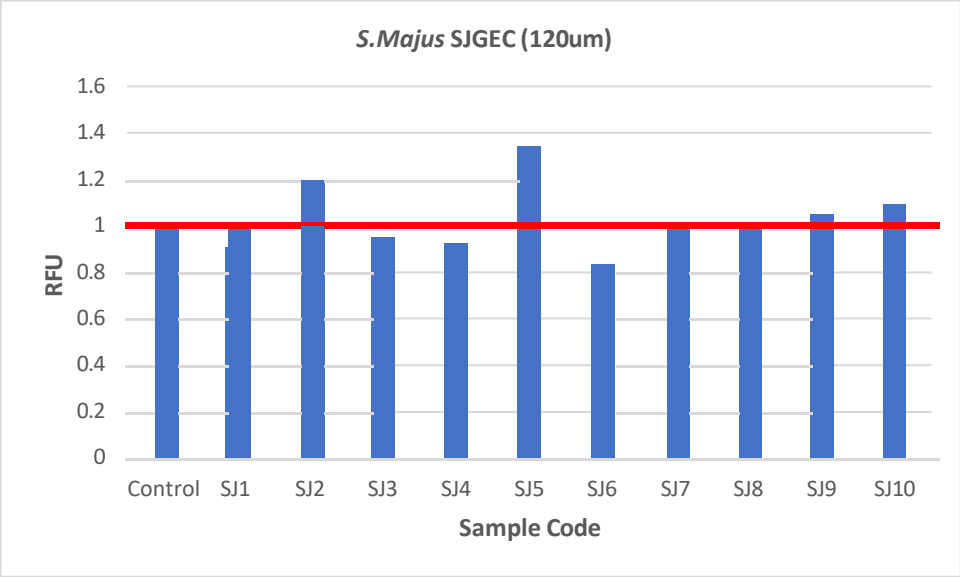


Figure 14 S. majus normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration.

S. majus outlined a mix response to the oryzalin treatment with SJ5 having a 34% increase in fluorescence, SJ2 had an increase of 21% whilst other samples displayed similar fluorescence to the control suggesting very little response to the oryzalin. Five samples provided results that were lower than that of the control. Average increase in fluorescence was 3% in the inoculated samples. Although average increase was relatively low *S. majus* provided a sample of interest (SJ5).

S. papillosum

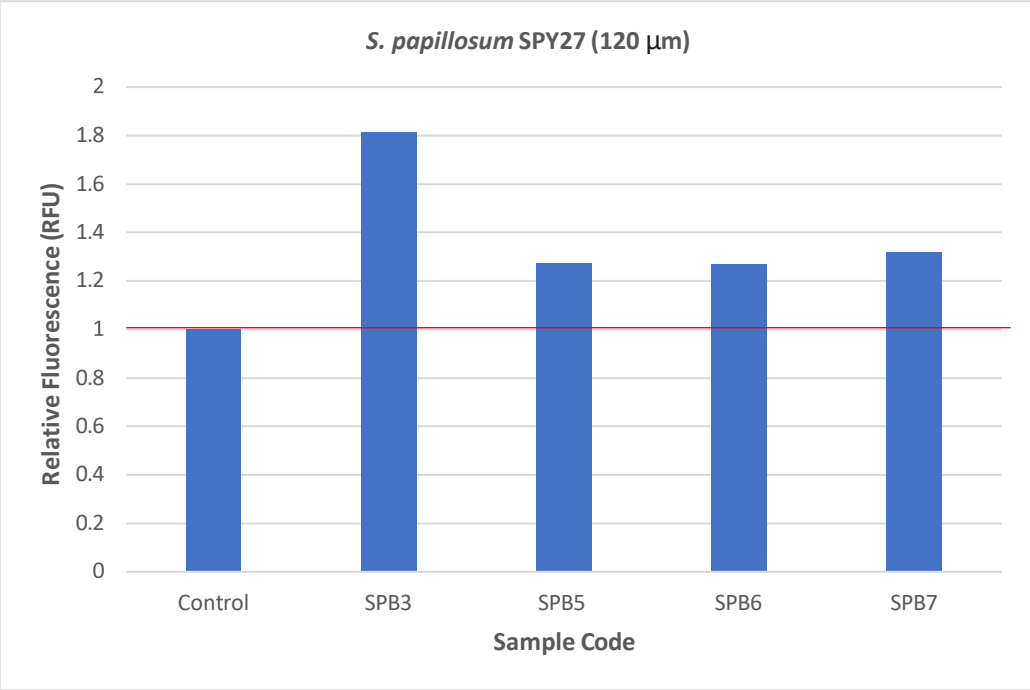


Figure 15 S. papillosum normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration

Findings from analysing S*. papillosum* would suggest a positive response to the oryzalin treatment. All samples provided a higher level of fluorescence than the control. Sample SPB3 had an increase in 81% suggesting a large portion of nuclei being arrested at a higher level of ploidy. Sample SPB7 also displayed a significant 32% increase in fluorescence. *Sphagnum papillosum* was one of the best preforming species in terms of reacting to oryzalin with a 45% average increase in fluorescence across samples.

S. russowii

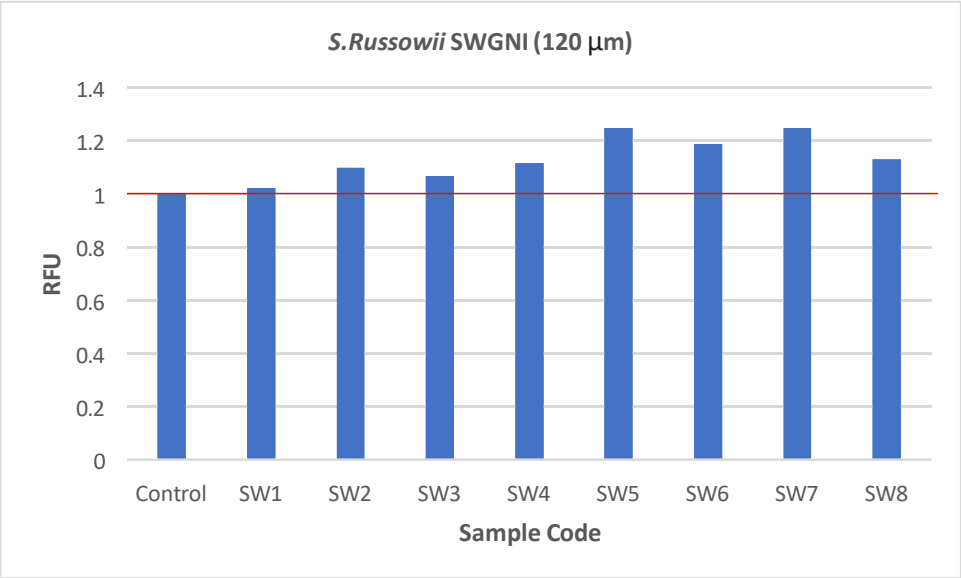


Figure 16 S.russowii normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration different sample codes outline different individuals within a species which have been used as a propagate.

Samples inoculated in *S. russowii* all displayed higher levels of fluorescence than the control. Average fluorescence was higher than the control by 13%. There didn't appear to be any samples of notable interest.

S. palustre

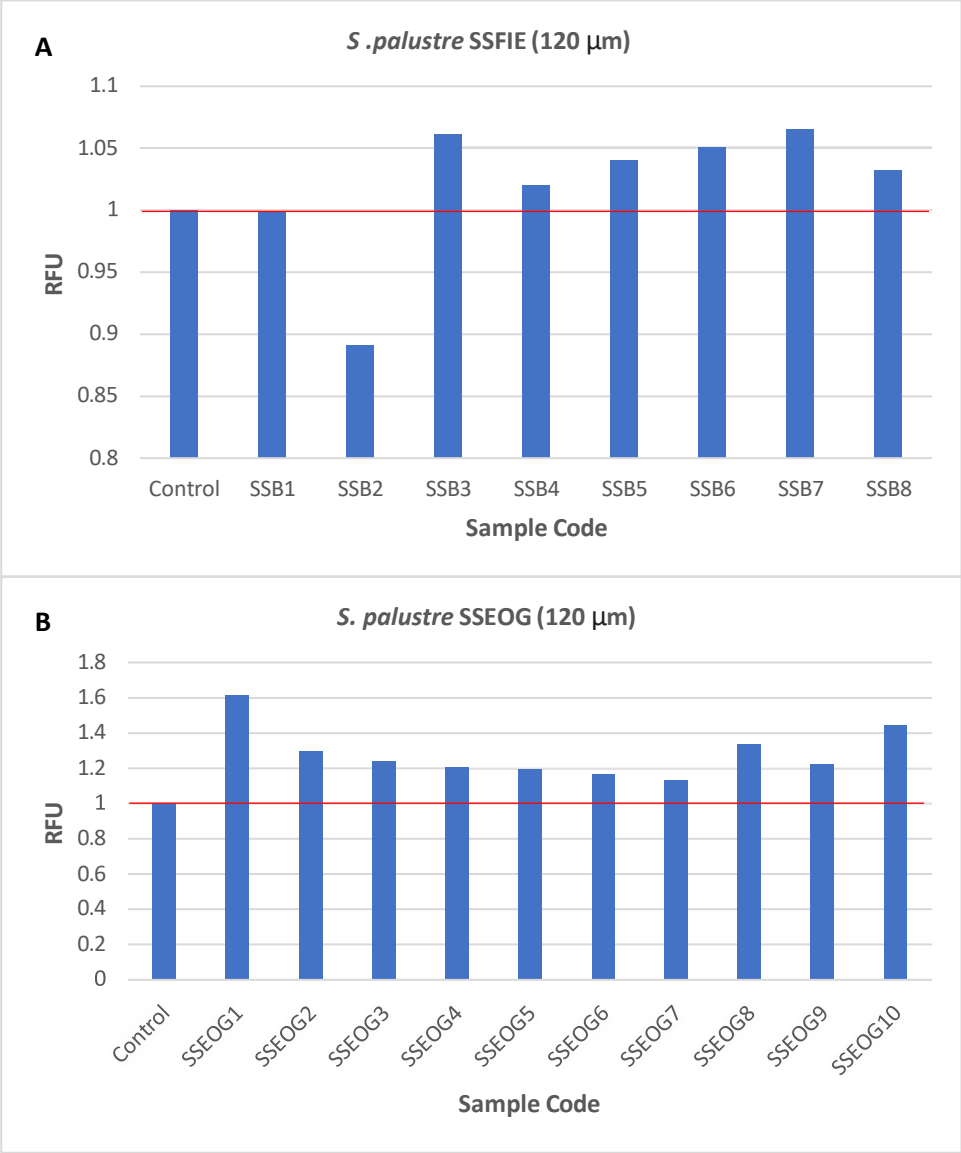


Figure 17 S. palustre normalised bar charts displaying propagated replicates from the same place of origin (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm concentration.

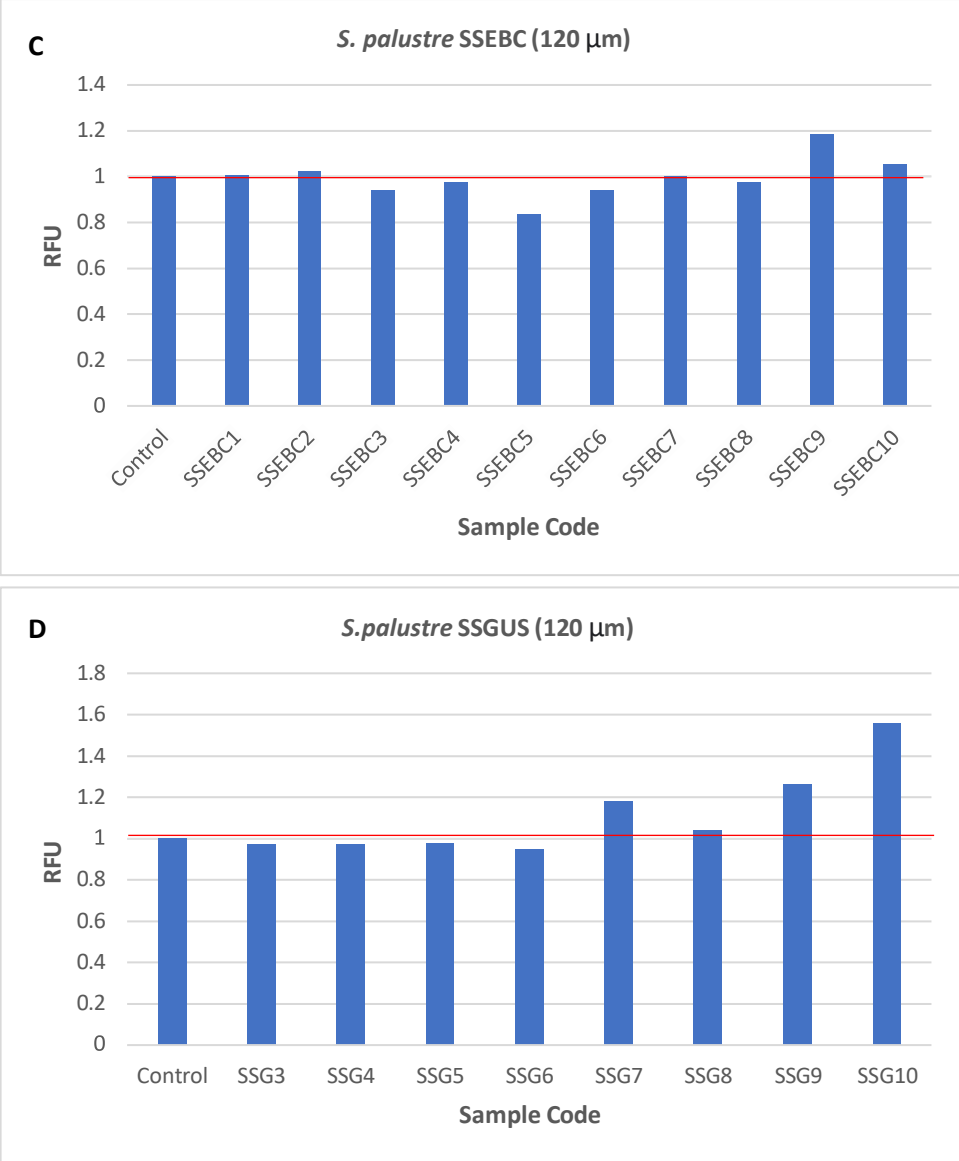


Figure 18 S. palustre normalised bar charts displaying propagated replicates (Sample Code X-axis) against relative fluorescence (RFU) (Y-axis). Samples are inoculated at the 120 µm *concentration.*

S. palustre analysis consisted of four different codes all inoculated at 120 µm Oryzalin. SSFIE **(A)** provided very little variance from the control and included a sample that fluoresced at a lower rate than the control (<12%). The batch did not present any novel individuals and had an average increase in fluorescence of 2%. SSEOG **(B)** provided three samples of novel interest (SSEOG1- 61%, SSEOG8- 34% and SSEOG10- 44%). All samples had higher recorded fluorescence than the control. Average fluorescence was 30% higher than the control. SSEBC **(C)** provided minimal variance after oryzalin treatment. Sample SSEBC9 provided the largest response to the treatment having a 19% higher fluorescence than the control. Average fluorescence of inoculated samples was .5% lower than the control. SSGUS **(D)** included one novel individual, (SSG10- 56% increase from control) SSG9 displayed a 26% increase when compared to the control. Average fluorescence was 11% higher than the control.

2.23 Performance Between the Same Species

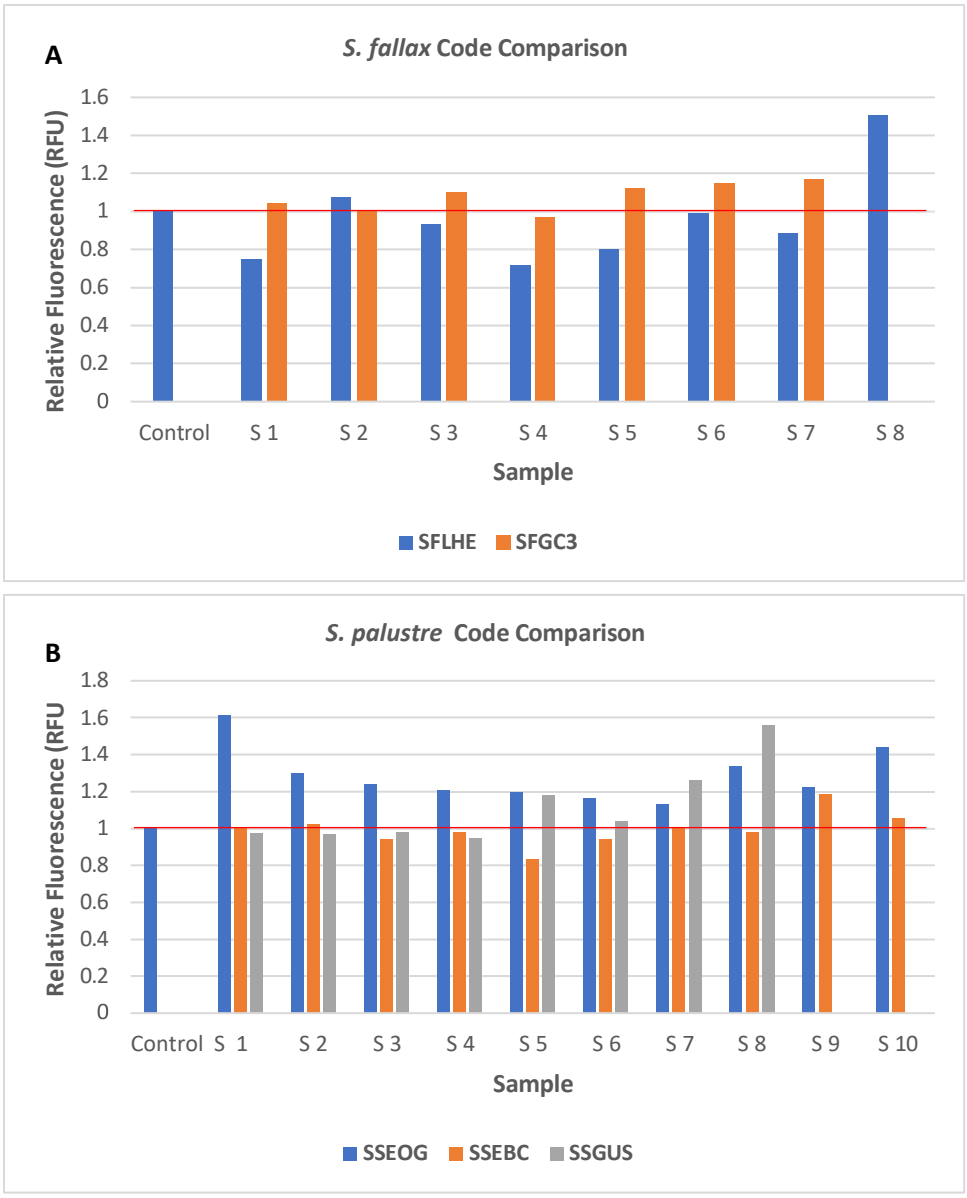


Figure 19 Comparative normalised bar charts highlighting effectivity of oryzalin between an individual species with different regional origins. (A- Comparison of two code batches in haploid S. fallax B- comparative of three code batches in the diploid S. palustre). Propagated replicates (X-axis) are compared against relative fluorescence RFU (Y-axis).

Comparisons between species codes could be made in the haploid *S. fallax* and the diploid *S. palustre* as these where the only two species containing multiple batch codes. *S. fallax* **(A)** consisted of two codes SFLHE and SFGC3. SFLHE provided the only novel individual sample and a large drop in relative fluorescence. SFG3 included the most consistency across inoculated samples potentially suggesting that *Sphagnum* of the same species *from* different regions can have entirely different responses to oryzalin treatment. *S. palustre* **(B)** Had a one clear batch code (SSEOG) that provided the most novel individual samples (3) and the most consistent level of increased fluorescence (30% higher than the control). SSEBC provided the lowest levels of fluorescence in terms of novel individuals (0) SSEBC also had an average fluorescence across samples closest to the result of the control suggesting it had the least response to oryzalin throughout the *S. palustre* codes analysed. SSGUS provided one novel specimen. SSEOG would appear to be the optimum *S. palustre* code to pursue further ploidy development work with using the current parameters and the current concentration of oryzalin.

2.24 Samples of Interest

Table 5 Species and specific samples that have shown a significant response to the oryzalin treatment. Samples were selected on a criteria of meeting 30%> increase in relative fluorescence when compared to a control. Kew reference values are present alongside the analysed estimated DNA amount. Ploidy level is also included as some samples have shown potential change.

Species	Code	Sample Number	Reference DNA Amount 1C (Picograms)	Estimated DNA Amount 1C (Picograms)	Ploidy Level
S. capillifolium	SAWBT (60 μm)	SAB1	0.46 (pg)	0.61 (pg)	Haploid
	SAWBT	SAB 6		0.71 (pg)	Haploid
S. fallax	SFLHE	SFL10	0.44 (pg)	0.65 (pg)	Haploid
S. squarrosum	SQGWB	SQ2	0.46 (pg)	0.91 (pg)	Potentially Diploid
		SQ3		0.63 (pg)	Haploid
		SQ8		0.65 (pg)	
		SQ9		0.61 (pg)	
S. majus	SJGEC	SJ5	0.81 (pg)	1.08 (pg)	Diploid
S. papillosum	SPY 27	SPB3	0.93 (pg)	1.67 (pg)	Diploid
S. palustre	SSEOG	SSEOG1	0.92 (pg)	1.48 (pg)	Diploid
		SSEOG8		1.23 (pg)	Diploid
		SSEOG10		1.38 (pg)	Diploid
	SSGUS	SSG10		1.43 (pg)	Diploid

As many samples inoculated with oryzalin displayed a positive reaction to the treatment, potential specimens of select interest were narrowed down to individual samples that had shown a 30% increase in fluorescence when compared to the standard.

S. capillifolium had two individuals of interest, one being the only specimen of interest from the lower concentration and one sample from the higher concentration. The sample from the lower concentration had increased fluorescence by 33% and the sample from the higher concentration had an increase of 60%. Both samples did not display an entire doubling and remain as haploid.

S. fallax had one sample of interest from the SFLHE code displaying just over 50% of all nuclei arrested at a higher fluorescence than the standard material. On a re-trial carried out in maturity It appeared that the sample had reverted to a measurement like that of the standard. Possible explanations could be due to an instability in the level of mixoploidy (mosaicism) and reverted to original value upon reaching maturity.

S. squarrosum appeared to have the largest response to the oryzalin treatment having four individuals of interest. SQGWB (SQ2) provided double the fluorescence of a haploid standard indicating a double estimated genome size. Although this is characteristic of a diploid the histogram peak is rather wide with high unacceptable coefficient of variance (CV%-29). This would suggest a diversification of fluorescence between the replicates in the sample which indicates that oryzalin is providing a fluctuations in nuclei populations in replicates., however also not desirable as it does not provide a clear population peak of all nuclei arrested in one stage.

In the diploid *S. majus* one sample displayed a 30% increase in estimated genome size and remains diploid.

S. papillosum sample SPB3 appeared to be very close in an entire duplication of genetic material, an increase of 80% in fluorescence in comparison to both the standard and the reference standard. A sample encroaching diploid nature it also displayed high CV peaks which would implicate instability within the sample.

S. palustre had samples of interest from two codes the first being SSEOG and the second being SSGUS. A good portion of the SSEOG samples displayed an increase in fluorescence between (34%- 61%). Samples also offered lower CV%s between (8-10%) accepted CVs are usually under 7%. Lower CVs would suggest that the samples are more stabilized with the oryzalin as the histogram peaks are narrower implying that all nuclei are fluorescing closer together along the channel. SSGUS sample SSG10 had 56% increase in channel fluorescence. Over half of the nuclei analysed displayed higher levels of fluorescence but not an entire migration to a polypoidal state. High CV also found in this sample once again suggesting instability with oryzalin.

2.25 Histograms of Doubling and Wide CV Peaks in Oryzalin Treatment

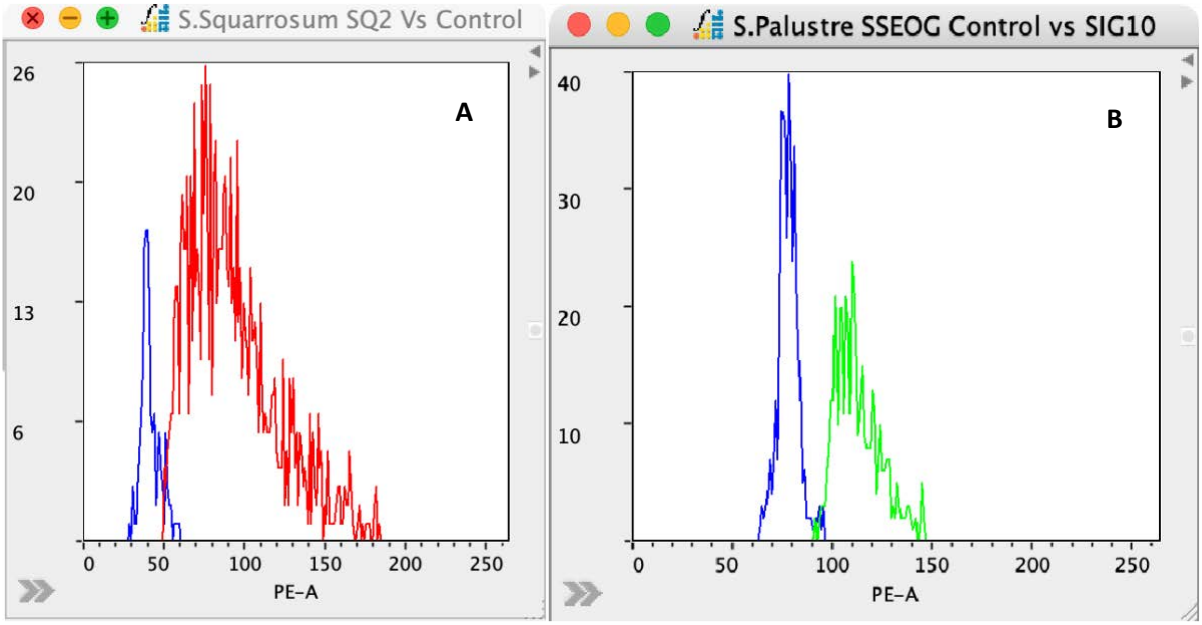


Figure 20 Fluorescence overlay histograms of specimens displaying high changes in fluorescence when compared to a control. Blue peaks represent the control sample. Red (haploid) and Red (diploid) indicate the inoculated samples. Channel fluorescence is displayed on the X-axis and the nuclei count is represented on the Y-axis. (A- haploid S. squarrosum against a control, B- diploid S. palustre against a control).

The width of peaks is measured by the Coefficient of variation (CV) and is an indication to how the populations of nuclei are distributed. A population with a very narrow peak would suggest a population of nuclei fluorescence at the same channel therefore all nuclei are arrested in the same position. It is evident from fig.19 that the control samples displayed thinner peaks than the controls. In **(A)** the inoculated *S. squarrosum* (red) has a wide CV (29%) which would not be accepted as a population. However due to the level of variance oryzalin induces to *Sphagnum* material it could be suggested. Its apparent despite that large variance that *S. squarrosum* Is fluorescing further down the channel implicit of nuclei with double the number of chromosomes. In **(B)** inoculated *S. palustre* (green) can be seen to have a peak double in width when compared to the standard material (Standard- 6.69%, SIG10- 12.29%). The standard peak (blue) appears lower down the channel than the '100' on the x-axis due to a voltage issue however still exemplifies that the induced material is represented at a double level of fluorescence indicating double the amount of genetic material in nuclei.

Discussion

Ploidy in *Sphagnum* has proven to be highly alternating between species, between propagated specimens and oryzalin inoculated specimens alike. Promising findings would implicate that synthetic application could increase levels of ploidy in *Sphagnum* samples. This study exemplifies that micro propagated *Sphagnum* has similar levels of DNA content to that of known standards at Kew. It also highlights how using the novel herbicide oryzalin at a concentration of 120um at 24 hours of exposure can provide nuclei of a mixoploidy nature and nuclei in haploid specimens of a diploid nature. Potential of future polyploidization is discussed incorporating the ideas of cell sorting and sub culturing specimens of interest.

2.26 Standard Material

The *sphagnum* samples used as an insight into levels of ploidy from micropropagation displayed the results anticipated. Estimated DNA amounts matched values outlined in the Kew c-value standards. This suggests firstly that the species used are the species thought. Secondly this would implicate that there isn't any large-scale change in nuclei ploidy level when going through an artificial growing process. Lastly this would suggest that the accuracy of the methodology would be correct as the estimated DNA quantities are aligning with that of Kew standards. This means that the mean peak channel readings observed and recorded are within the boundaries of the standard implicating the method would be successful in future study. There was one sample standard which did not provide a reflective result. *S. denticulatum* provided a haploid reading but not of the correct estimated DNA size (recording- 0.61 pg) standard 0.39 pg). This can be implicit of either improper instrument alignment (Dolezel et al., 2007) or more likely due to all other species providing credible results a sample that could be a different species. The only reference with a value within (0.05 pg) is *Sphagnum recurvum* (0.56 pg). Clarifying if the sample is *S. recurvum* may be potentially difficult as a complex is apparent in which identification in *S. recurvum* is conflicted between one polymorphic species and a complex describing 6-10 species (Duffy et al., 2020). The use of DNA sequencing could be an effective tool to pinpoint the identification of this sample.

2.27 60 μm Vs 120 μm Concentrations

The concentrations of oryzalin used provided drastically different results. What was thought to be two concentrations of the herbicide optimized for haploid samples (60 μm) and the second for diploid samples (120 μm) displayed quite the opposite with 120 μm providing the gratest range in relative fluorescence in both ploidy types analysed. In the haploid S*. capillifolium* oryzalin had more of a response in samples inoculated with 120 μm. Average fluorescence was 27% higher than the non-inoculated and 20% higher than the 60 μm concentration. This suggests that haploid *sphagnum* samples are a lot more tolerable of higher concentrations of oryzalin than originally expected.

In the diploid samples the 120 μm concentration provided the best response to oryzalin as expected. Comparatively, haploid samples displayed higher concentrations of fluorescence when compared to a control than the diploid. This could implicate that higher concentration thresholds of oryzalin could be potentially used in the diploid samples. Although 120 μm has seen to be a common place to induce polyploidy in flowers and fruits (Ascough et al.,2008, Contrerars, 2009) very little is understood in what concentration is palatable for active encouragement of polyploidy in *Sphagnum*. In shrubs such as *Hebe oriatia* successful inoculation has been seen using higher levels of oryzalin (289 μm) (Gallone et al.,2014). *Hebe oriatia,* also harbor a relatively small genome sizes (0.26 pg) (Meudt et al., 2015) like that of the *Sphganace* this would suggest that species with smaller genome sizes possibly need larger exposures of oryzalin to increase levels of ploidy.

The concentration at 120 μm has provided a range of varying individuals some of which providing far higher levels of fluorescence than the 60 μm concentration and the standard material. Oryzalin can be seen to be having a substantial effect at these concentrations and is representative of a mixoploid population. Perhaps to a achieve a complete increase in chromosome doubling higher concentrations of oryzalin are needed, to what degree exposure is sufficient will be very fine as over exposure will result in destruction of intact nuclei.

2.28 Batch Codes of the Same Species

Micropropagation services have many different places of origins in which micro propagated material is procured from. Most species are taken from various sites across Europe. The possibility to analyse multiple different origins allowed for intraspecific analysis in the haploid *S. fallax* and the diploid *S. palustre.* The possibility of the same species extracted from different regions having different responses to oryzalin seemed to be highly prevalent. *S. fallax* consisted of two codes SFLHE and SFGC3 (Fig.19). SFLHE provided the only novel individual replicate however observed a negative response to inducement with most replicates having lower RFU. SFG3 included the most consistency across inoculated samples potentially suggesting that *Sphagnum* of the same species *from* different regions can have entirely different responses to oryzalin treatment. Things to consider here are that the region itself may not be anything to do with the fact that oryzalin response was better. This could simply be due to how incorporated the oryzalin became with the samples at the time of treatment. As the samples are sterile and suspected to be at the same level of ploidy.

In the diploid *S. palustre,* four different codes provided three very different results. SSEOG would appear to be the optimum *S. palustre* code to pursue in further subculturing and ploidy development work (Fig 19). This is due to the code having three novel samples across 10 samples analysed. This exemplifies the benefit of using of multiple codes from one species, the method lessens the rarity of novel specimens. For the next screening of material, it will be vital to incorporate more codes from same species to highlight any potential increase in ploidy. Micropropagation can take the prime codes compared SFLHE and SSEOG and focus on those individually plating up further propagates and inoculating with varying new thresholds of oryzalin.

2.29 Oryzalin Receptive Samples

One of the objectives of this chapter was to explore the possibilities of polypoidal *Sphagnum.* It wouldn't appear that polyploidy in *Sphagnum* has been achieved by the means of oryzalin inoculation. The proportion of polyploidy in mosses naturally is low (5-10%) (Stenøien et al. 2010) with the majority of polyploids being consequence of allopolyploid (via hybridization), (Sastad, 2004). This raises the question if *Sphagnum* is polypoidal. Allopolyploidal *Sphagnum* is prevalent across the southern hemisphere as *S. australe* and *S. falcatulum* (Karlin et al.,2009) and in *S. subsecundum* (Ricca et al., 2011).

In contrast autopolyploid is presently non-existent in *Sphagnum.* This study provides a compelling start to achieving such a state. A first of its kind achievement in the *Sphagnum* genus, an entire duplication of *S. squarrosum* can be seen in (table 5) and a fluorescence peak double that of a standard can be seen in Fig 20. This study outlines oryzalin's success of increasing levels of ploidy in haploid specimens. It can be argued that this is not a stable ploidy increase as the population peak displayed high CVs, but still a haploid turned diploid specimen. *S. squarrosum* could potentially find meiotic defects with its newfound ploidy level which can result in reduced fertility due to aneuploid gametes (Gonzalo 2022). This could potentially provide the opposite effect desired in a *Sphagnum* farming scenario. To avoid such an issue, specialized meiotic programs known as cytological diploidization have seen to 'evolve' consequent meiotic irregularities in a number of higher ploidy plant studies (Grandont et al.,2014, Morgan et al.,2021). Applying a program such as this would allow for synthetically increased samples to gain versatility and stability when applying to *Sphagnum* farming situation. If the ability to increase ploidy levels persists in *Sphagnum* adopting a practice such as this will enable a high throughput of enhanced samples for *Sphagnum* farming.

Other Haploids such as *S. capillifolium* and *S. fallax* display higher estimated DNA amounts after inoculation with oryzalin, these species did not reach a level of diploid like *S. squarrosum.* This would suggest microtubule assembly disruption Is occurring (Ascough 2004) and cellular division is being stopped. The question being if the oryzalin is actively encouraging replication of genetic material and inhabiting cellular division why, is an entire duplication of ploidy level and DNA not apparent. This again could be explained by treatment, its suspected that longer oryzalin treatments can increase diploidization of a larger number of cells and reduce the rate that oryzalin creates mixoploids (Tosca et al., 1995). Longer treatments have often proven to be toxic in plants (Telebi et al., 2017) but their effects in *Sphagnum* are not yet understood. *Sphagnum's* ability to tolerate herbicides could vary massively from species to species. *Sphagnums tolerance* could be higher than plants previously inoculated with oryzalin. Analysis on samples of higher thresholds would be a good insight into if a complete jump to a diploid level could occur in *S. capillifolium* and *S. fallax.*

Similarly, diploid species highlighted similar mixed results to that of the haploid species. *S. papillosum* provided the closest specimen to reaching a polypoidal state. Mean nuclei fluorescence was 80% higher than the standard suggesting that most nuclei were arrested at a higher ploidy level. This achieved a mixoploid ploidy level and has been seen to be more common than making tetraploids (polyploidy) when using herbicide inducements (Ascough et al., 2008). Achieving a polypoidal state in *Sphagnum* could potentially be challenging when compared to other mosses, as *Sphagnum* is one of the only mosses that displays no evidence of endopolyploidy (Bainard et al., 2010, Pa'lova et al., 2020).

Endoreduplication occurs naturally in somatic cells of most mosses and results in a modified cell cycle creating a nucleus with double the DNA content (D'Amato, 1964), a naturally occurring small amount of polypoidal cells. Around 90% of angiosperms have evidence of endopolyploidzation (Scholes et al., 2015) which could explain their large success with oryzalin (Touchell et al., 2020, Mo et al., 2020).

Sphagnums lack of endopolyploidy suggests there are no polypoidal cells being formed by any means naturally, putting *Sphagnum* at a disadvantage in trying to actively form polypoidal nuclei. The best possible means of achieving polyploidy in *Sphagnum* could be through subculturing samples of interest to identify if there is any generational progression or more novel techniques such as cell sorting.

2.30 Polyploidy Through Sub Culturing and Cell Sorting

The possibility of polyploidy in *Sphagnum* is not unrealistic. Specimens outlined in this study have shown a relationship with oryzalin is apparent. It might be the case that *Sphagnum* material may just need an extra level of consideration due to its small genome size and its lack of endopolyploidy. The usage of micropropagation in this study promotes the novel situation of propagating again. An Autopolyploid state in *Sphagnum* may be achievable through subculturing to encourage desired levels of ploidy. Taking samples of haploid and diploid nature that have shown considerable increase in mean fluorescence and estimated DNA amounts and sub culturing following the same oryzalin treatment could potentially prove to be successful. A double treatment procedure could be what it takes to create a polypoidal specimen. Although most oryzalin treatments call for only one round of treatment (Kermani et al., 2003, Švécarová et al., 2019) most material derive from seeds or spores. This study is unique as its one of the only invitro polyploidy inducement trials using material from a micropropagated source. The possibilities of achieving polyploidy by multiple trials and propagations could be a means to integrate auto -polyploidy in species which prove difficult to induce by current methods.

A second possibility to achieve polyploidy could be via the means of cellular sorting. In combination with flow cytometry, Fluorescence activated cell sorting (FACS) might be able to separate desired nuclei of interest. Cell sorting allows for sorting of sub populations and provides a more in-depth characterization of cells with select properties obtained from flow cytometry (Mattanovich and Borth, 2006). The application of this to *Sphagnum* would allow for the isolation of sub-populations of nuceli with different ploidy levels. Nuclei are primarily sorted in flow cytometry establishing individual populations, once these populations are isolated, cell sorters apply a charge to a desired population (Garavello et al., 2019). A fluorescence labelling marker is also added to distinguish populations of interest (Galbraith, 2012) so in this case to nuclei that have displayed higher levels of fluorescence. FACS allows for subpopulations to remain intact for further use. If a nuclei suspension protocol can be devised that does not permeabilize (fix) nuclei and a protocol can be developed to sort nuclei using FACS, it's very possible that isolated suspensions consisting of solely nuclei with higher DNA amounts could be obtained and plated creating *Sphagnum* with no residual ploidy levels. All things considered, a study such as this would take a monumental amount of time and investment and should be a secondary consideration if subculturing is unfruitful.

Conclusion

Sphagnum has exemplified its complex nature throughout the course of this chapter. To understand polyploidy, it was first needed to understand ploidy. Material grown at Beadamoss via micropropagation has the same ploidy found in reference standards as the Kew C-values. An understanding of a wide variety of *Sphagnum* species ploidy are now recorded and can provide as a standard for future research. All species analysed displayed the level of ploidy anticipated expect *S. denticulatum* which is suspected to be another species *S. recurvum.* Attempting to induce polyploidy has not been as successful as first hoped however this study has identified the first ever increase in ploidy level using oryzalin in *Sphagnum* from *S. squarrosum* becoming a diploid sample. On a whole *Sphagnum* did display positive responses to oryzalin providing novel samples of interest. Through the means of subculturing said samples of interest the potential for polyploidy in *Sphagnum* could become a possibility.

Chapter 3-

Ploidy levels across Species of Origin

Introduction

Ploidy in plants has been seen to display high levels of variance with some plants having higher numbers of chromosomes in different regions of the world (Robinson et al., 2018) and has also been seen in *Sphagnum* (Stenoien et al., 2010). It has been identified that community level intraspecific variation in ploidy provides the strongest indicator of plant community species richness, plant species distributions over fragmented landscapes, and local extinction indication (Plue et al., 2018). Understanding these regional differences could prove crucial for bettering understanding of such interactions in ecosystems and potentially uncover novel species with unseen ploidy levels in other parts of the world.

In mosses generally, increased levels of ploidy have been shown to be a significant process in evolution (Shaw et al., 2016) often associated with hermaphroditism and influences on sexual systems, and mating patterns (Jesson, 2011). The issue with many mosses' species in terms of progressive ploidy development is their fixed heterozygosity state (Alix et al., 2017). Fixed heterozygosity creates a fixed number of chromosomes per cell. Fixed heterozygosity has been seen in *Sphagnum* and has been consequential of genetic drift, and a suspected loss in genetic diversity as a founder effect from postglacial recolonisation of northern Europe (Thingsgaard, 2001). (Thingsgaard, 2001) also pointed out that *Sphagnum* was undergoing a decrease in genetic diversity latitudinally. This has been made apparent in *Sphagnum* studies several times including *Sphagnums* lack of endopolyploidy (Bainard and Newmaster, 2010) and its continual display of unequivocally haploid (19 chromosomes) and diploid (38 chromsomes) samples when analysed in 19 different species of *Sphagnum* (Heck et al., 2020).

Interestingly, latitudinally Sphagnum has been seen to have a higher genetic diversity with the findings of hybridized allopolyploids located in northern Norway (Stenoien et al., 2010) and the allotriploid *S. falcatulum* New Zealand as a result of the hybridization of three monoploid genomes of three ancestorial species. (Karlin 2017). In the long scheme, testing more of these individuals from polarised latitudinally could result in finding samples that have surpassed a fixed heterozygotic state from non-hybridized specimen.

This study investigates micropropagated non hybridized *Sphagnum* from a multitude of regions in the UK and Central Europe. The importance of highlighting the ploidy of these specimens come at a benefit of being able to regionally pinpoint levels of ploidy which can help sustain and colonize habitats (Beest et al., 2011) such as peatland rejuvenation sites. Secondly this can provide a greater agronomic benefit when undertaking carbon farming using *Sphagnum* material of higher ploidy levels by similar means to peatland rejuvenation sites through a more efficient establishment process and a wider robustness resulting in a higher toleration of invasive species and a reduced susceptibility to disease (Sattler et al., 2016). Expectations of ploidy in samples analysed are considered to see increased genetic content of haploid species potentially being diploid and even possibly diploid species showing polyploidy. The objectives of this chapter

The objectives of this chapter are:

- To regionally pinpoint specimens of notable genetic diversity by measuring relative fluorescence, determining genetic content.
- Correlate notable specimens, assessing regional relationships between various species in the sphagnum genus.

Methods

3.1 Sample material

Species of Origin Samples		
Species	**Species Code**	**Place of Origin**
S. denticulatum	SDENT	Peak District
	SDGWE	Germany
	SDSRM	Scotland
	SDY28	Yorkshire
	SDY34	
S. fallax	SFBLT	Peak District
	SFHOL AC	
	SFMGW G	
	SFY10	Yorkshire
	SFYEA	
	SFHSS	Northern Ireland
	SF6F6	Germany
	SFLHE	Latvia
	SFW5E W	Shropshire
S. palustre	SSMHS	Peak District
	SSGFO	
	SSHTE	
	SSSRE A1	
	SSSTW-V	
	SSRE-F	
	SSGET	
	SSHYN	Yorkshire
	SSY31	
	SSGUS	Germany
	SSR6-F	
	SSR4D	
	SSWO5	Shropshire
	SSWR8	
	SSAB	Scotland

Table 6 Species of origin sample contents. Three species have been analysed. Haploid, S. denticulatum, S. fallax, and diploid S. palustre. Species code and their place of origin are also specified.

Samples required for this study needed to be of different regionality to detect if there was in fact a difference in ploidy levels between different locations. To achieve this Beadamoss Micropropagation Services provided samples from across the UK and Northern Europe. The species included in this trial *S. denticulatum, S. fallax* and *S. palustre.* This would allow for observable differences in both haploid and diploid *Sphagnum.* Species were analysed across a total of seven different locations (Table 6) with some locations having multiple samples from said location.

3.2 Extraction

Sample material was analysed following the procedure refined in chapter 1. *Sphagnum* material was lightly chopped in 1 ml of LB01 lysis buffer in a titled petri dish. The suspension was then homogenised via pipetting three times whilst actively avoiding the creation of bubbles. One final 1 ml of the liquid suspension was then passed through a 0.42um nylon mesh filter into a flow tube. The sample was then incubated on ice for 10 minutes. Rnase (a) added at 0.50 ug and Propidium Iodide (PI) added at 150 ug were incorporated into the sample simultaneously before flowing cytometric analysis. (See Final procedure – reference number).

3.3 Acquisition and Post Analysis

Upon readying samples for analysis, initial procedure checks were carried out the same as (Chapter 1- preparation of flow cytometer). Samples where then analysed for levels of fluorescence using a BD FACs Celesta. Initial acquisition on the abscissa to determine peak positioning was done with *S. fallax* as it is one of two species from the *Sphagnum L.* genus to have a genome sequence publicly available. Haploid peaks where set to a channel volume of 50 and recordings occurred up to 30,000 events on the medium flow setting. Data recorded included fluorescence histogram peaks including the mean, median, and Coefficient of variance. Acquisition of nuclei was achieved using the procedure outlined in Chapter 1 (reference number). Once an entire data set had been attained from the flow instrument, data was further analysed using Flow Logic to interpret mean fluorescence values and aid in forming normalised data.

3.4 Data Representation

Data was presented predominantly in normalised bar charts. As the histogram peaks provided rather broad peaks in some cases this was the most interpretable means of visualising differences between inoculated samples and the controls. This was presented as a control displaying the value of (1) for its 1C value.

Results

Species compared in codes were outlined using the normalised bar chart representation found throughout the entirety of the study. In which, the mean fluorescence value was taken for each code. Each code was represented by a single bar and measured in relative fluorescence units (RFU). A control code that featured micropropagated material used in the preliminary methodology study, with a now known ploidy level was used to highlight changes in levels of ploidy between codes and origin of material. Expected haploid species analysed included S. *denticulatum* and *S. fallax*. Expected diploid species analysed included *S. palustre.*

S. denticulatum

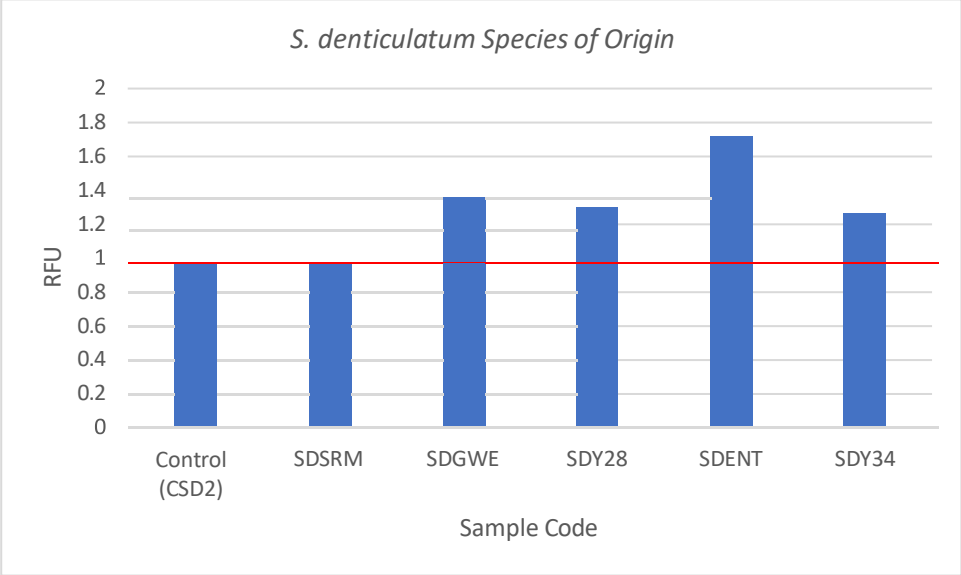


Figure 21 Species of origin comparison of S. denticulatum using a normalised bar chart. Place of origin is represented by each Sample code and are indicated by blue bars (x-axis), measured in terms of relative fluorescence units (RFU) (Y-axis). Red line indicates the RFU value of the control.

S. denticulatum consisted of five separate species codes with origins from Yorkshire, Scotland, Germany, and the Peak District. Species codes in *S. denticulatum* did not indicate any direct higher levels of ploidy between different places of origin. SDENT provided the highest RFU value with a 70% increase in fluorescence when compared to the control. SDENT originated from the Peak District. SDGWE also displayed an increase of 40% fluorescence when compared to the control originated from Germany. The lowest relative fluorescence (SDSRM) originated from Scotland whilst SDY28 and SDY34 displayed a similar level of fluorescence both originated from Yorkshire.

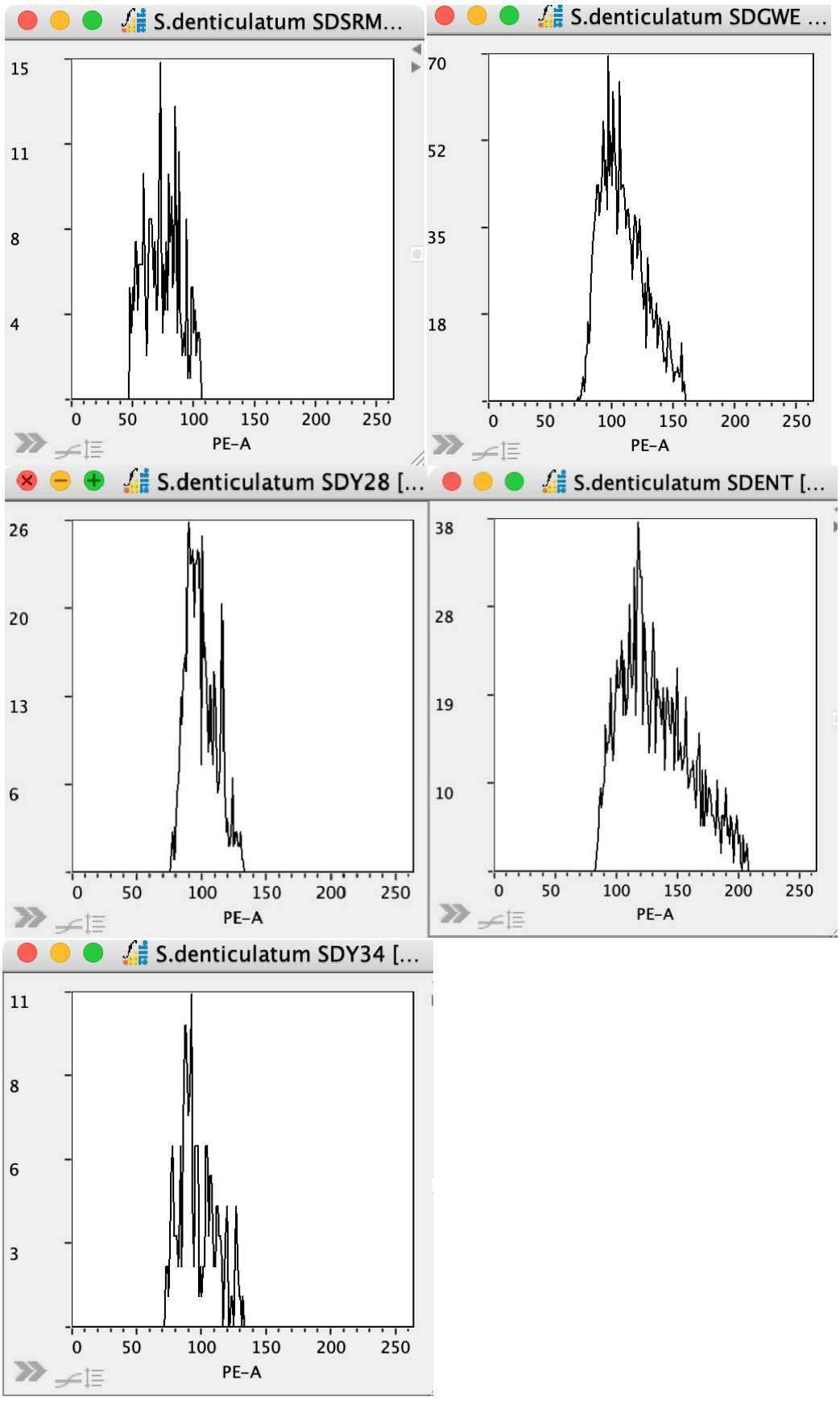


Figure 22 Flow cytometry histogram plots of diploid S. denticulatum with channel fluorescence (PE-A) on the x-axis and nuclei count on the Y-axis.

S. fallax

S. fallax consisted of seven different codes originating from Yorkshire, Shropshire, Latvia, Northern Ireland, and Germany. In *S. fallax* one code displayed double the amount of fluorescence than that of the control indicating *S. fallax* of a diploid nature. Sample code SF6F6 originating from a German source appears to be diploid. All the other codes analysed provided very similar levels of fluorescence to that of the control in the codes indicating that most origin locations have haploid *S. fallax*. In (SFHSS- Northern Ireland, and SFYEA- Yorkshire) fluorescence was lower than that of the control.

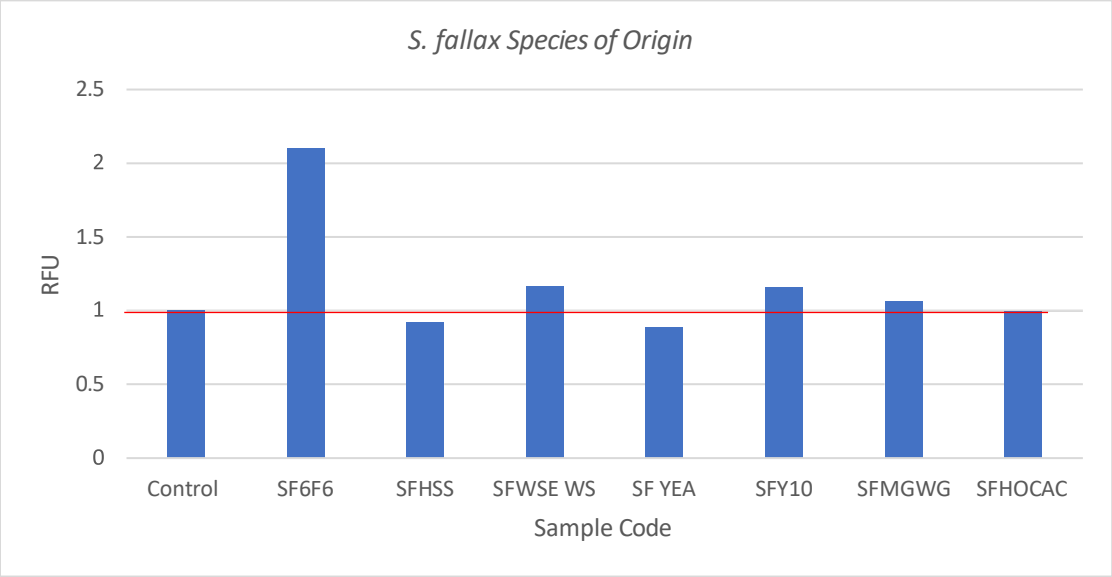


Figure 23 Species of origin comparison of S. fallax using a normalised bar chart. Species regionality is represented by Sample codes indicated by blue bars (x- axis) and are measured in terms of relative fluorescence units (RFU) (Y-axis). Red line indicates the RFU value of the control.

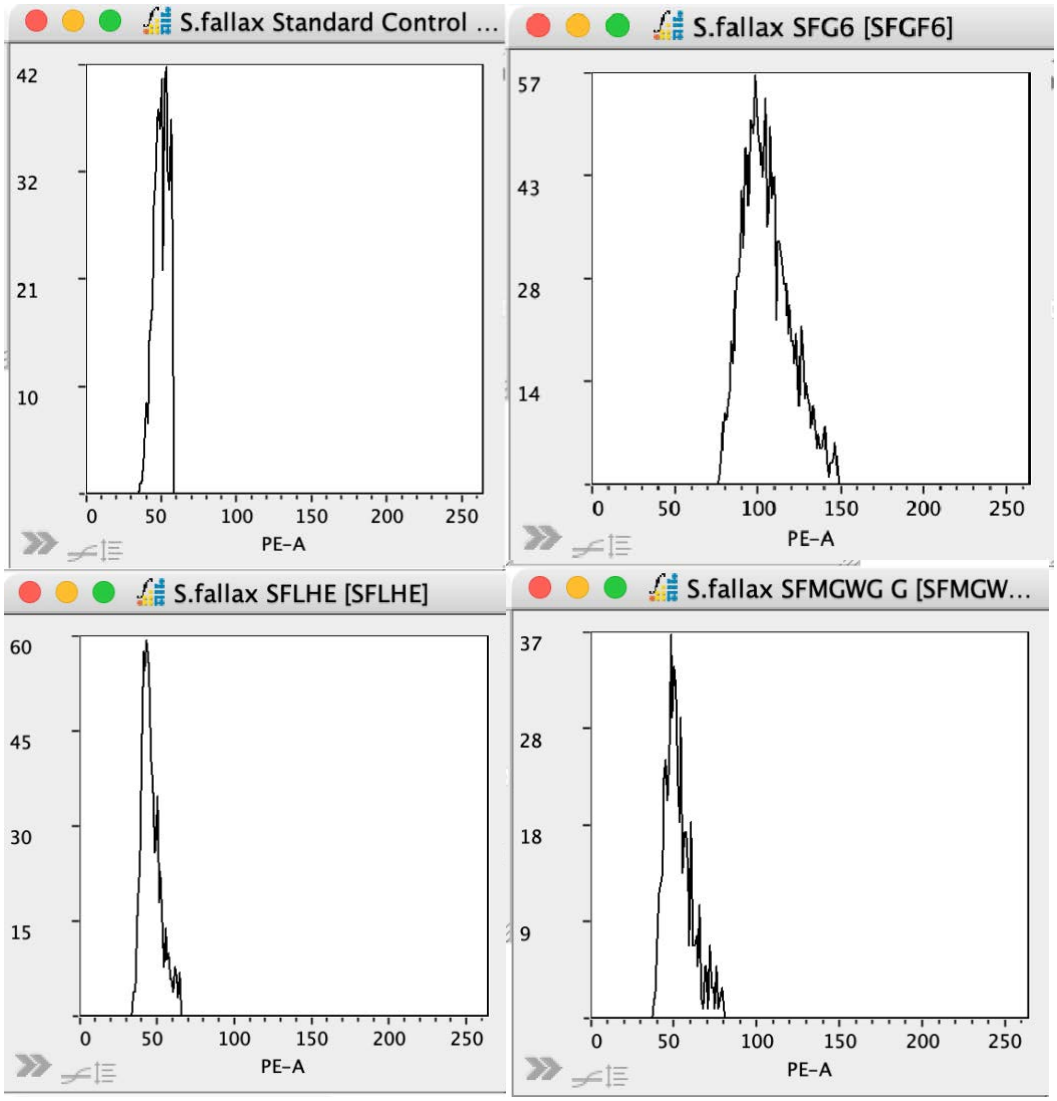


Figure 24 A selection of flow cytometry histogram plots of haploid and diploid S. fallax with channel fluorescence (PE-A) on the x-axis and nuclei count on the Y-axis.

S. palustre

S. palustre consisted of fifteen individual species codes including Eire, Wales, Germany, Latvia, Yorkshire, Shropshire, Exmoor, Scotland, and the Peak District. *S. palustre* is diploid and most codes of origin displayed similar levels of fluorescence to that of the control. One code SSGFO (Peak District) displayed a particularly low fluorescence, 61% lower than that of the control possibly suggesting that this code could be a different species. A natural occurrence of polyploidy did not appear throughout the analysis. Variance appeared to be much higher in the haploid samples, a finding that has been apparent throughout each chapter. Diploid samples across origins remain arrested in a diploid state.

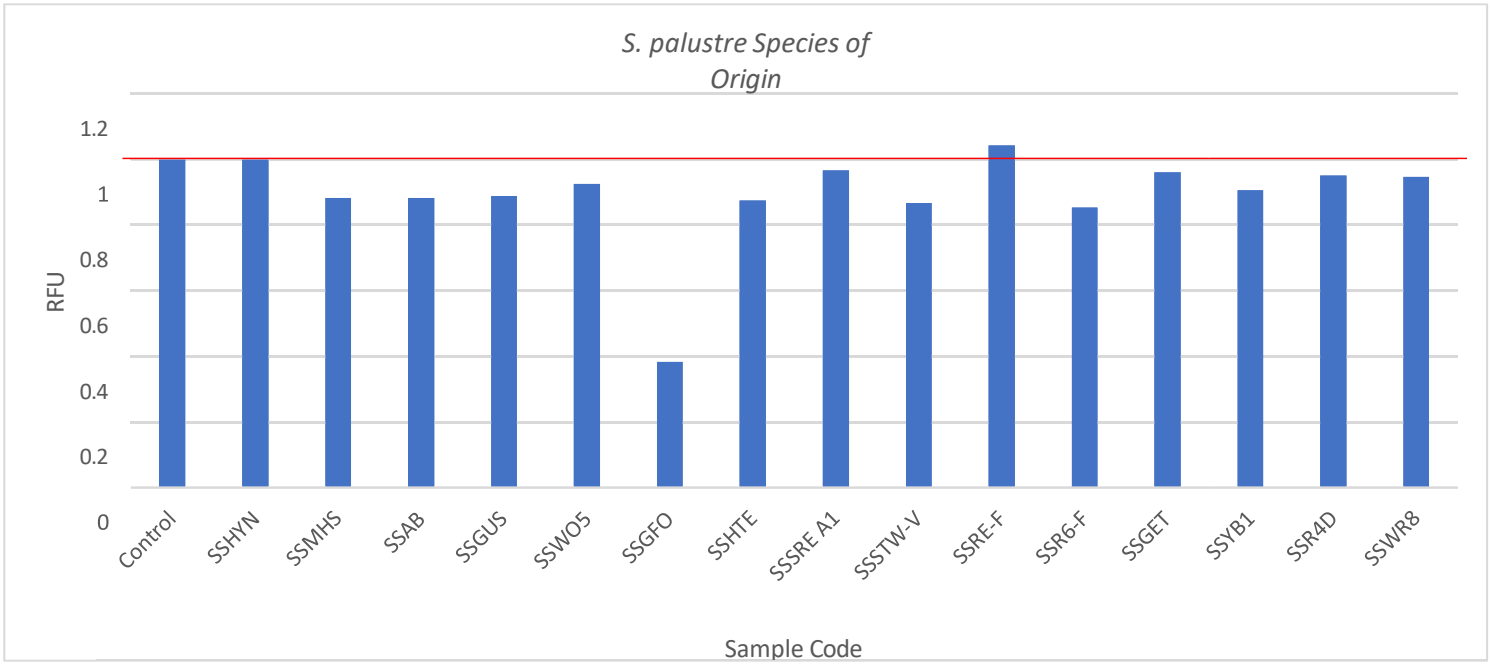


Figure 25 Species of origin comparison of S. palustre using a normalised bar chart. Specimens region of origin is represented by the Sample codes indicated by blue bars (x-axis) and are measured in terms of relative fluorescence units (RFU) (Y-axis). Red line indicates the RFU value of the control.

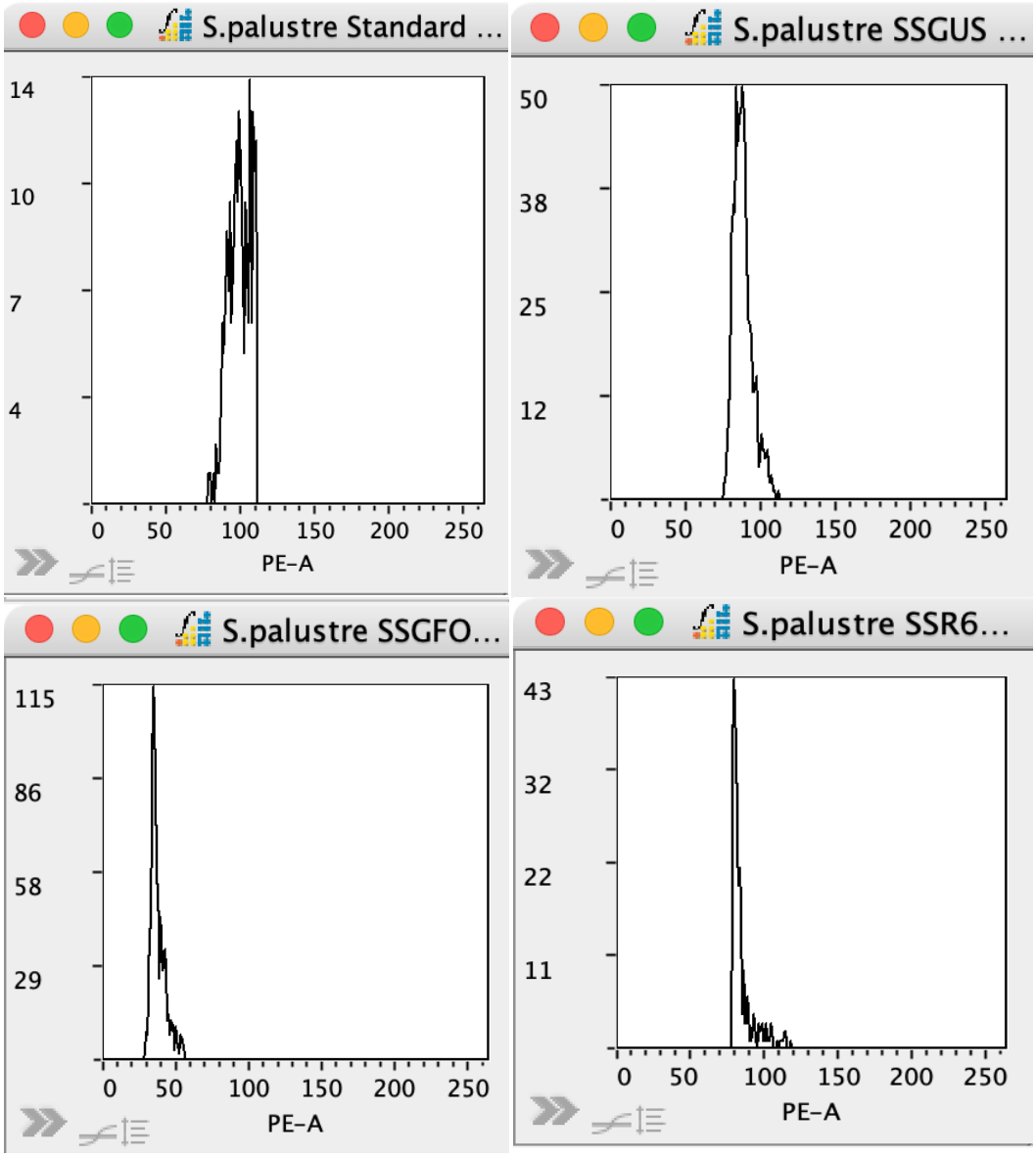


Figure 26 A selection of flow cytometry histogram plots of diploid S. palustre and a histogram of a potentially different species at a haploid level (SSGFO). Channel fluorescence (PE-A) on the x-axis and nuclei count on the Y-axis.

Discussion

This study was carried out to better understand regional differences in ploidy across haploid and diploid *Sphagnum* species. What is evident is that regional variance in ploidy did not appear to be as vast as suspected. In most of the species analysed, ploidy levels remained the same in *S. denticulatum* there appeared to be a larger amount of variance in mean channel fluorescence (*S. denticulatum*) in some codes than others, in particular codes that had species from Germany and the Peak District. This could indicate that said locations could be seeing samples of a mixoploid nature. This is difficult to deduce analytically as *Sphagnum* is haplophasic (Heck et al., 2020). When using flow cytometry *Sphagnum* specimens appear with only one single peak. This makes it problematic to see a conventional cell cycle and determine cells that are arrested in smaller populations through flowcytometry. This could be consequential of *Sphagnums* small genome size (0.47 bp) (Shaw et al., 2016) resulting in particularly low nuclei counts. Specimens from these places of origins, however, would be beneficial for further micropropagation, they clearly show higher levels of channel fluorescence indicating cells of a greater complexity and could potentially pose for an increase in ploidy in time.

In *S. fallax* one sample from a German origin displayed a diploid reading (figure 24). SF6F6 displayed a channel volume represented at a diploid nature, this would suggest that there is a more apparent amount of diploid *Sphagnum fallax* in Germany. The reason for its apparency could be explained by the trend of *Sphagnums* genetic diversity decreasing as longitude increases (Thingsgaard, 2001). Regional explanations of increase in cytotypes can be attributed to multiple processes including the evolutionary history of *Sphagnum.* Other process includes dispersal capabilities, biotic factors such as competition, and abiotic factors such as temperature and precipitation (Levin, 2002; Lexer and Van Loo 2006). It is rarely understood which combination of process create such distributions of variance in terms of cytotype (Castro et al.,2020). What can be inferred is that having a known natural diploid individual of *S. fallax* can pose to be advantageous from a carbon farming perspective and from a peatland rejuvenation perspective. Integrating a diploid *S. fallax* poses for better rates of establishment in both practices due to increased physiological traits such as thicker epidermis (Maherali et al., 2009), wall thickness and lignin content (Hao et al., 2013). Physiological changes result in changes in gas and water transportation resulting in increased levels of drought tolerance, influencing niche expansion (Manzenda et al., 2012).

In *S. palustre* very little was seen to change in terms of ploidy regionally even though it had the largest number of species of origin investigated. The lack of polyploidy would suggest that the results found support the notion of fixed heterozygosity in natural non hybridized Sphagnum populations. The results of the diploid *S. palustre* maintain the principal finding of the *Sphagnum* L. genus being unequivocally haploid or diploid naturally. Taxonomic groups such as liverworts and hornworts have been seen to have a divergent lineage when it comes to polyploidy (Weiss-Schneeweiss, 2013) although multiple whole genome duplication (WGD) events have been outlined in *Sphagnodopsia.* The most recent WGD predates divergence of *Sphagnum* from two other genre of *Sphagnodopsia* (Devos et al 2016). This would suggest that the plausibility of non-hybridized *Sphagnum* going through a (WGD) event to achieve polyploidy in the likes of species such as *S. palustre* is possible but would need specific conditions and optimum functional processes.

Allopolyploid (multiple sets of chromosomes from multiple species) are prevalent in *Sphagnum* (Karin et al 2009, Ricca et al 2010). Allopolyploidy has been seen to be most frequent in monoicous species such as sphagnum, that grow in 'poor' conditions with low pH's that produce relatively small spores. (Melashenko et al., 2018). The use of an allopolyploid sample would have been useful within this study to understand how a polypoidal reading functioned with the flowcytometry. Obtaining and analysing material such as this could be useful when analysing polyploidy whether it be from natural sources or from synthetic induction. Micropropagating species such as this could also prove fruitful from an archive perspective. The role of allopolyploidal species in *Sphagnum* farming has not yet been considered in academic literature but would hopefully provide a useful alternative to an autopolyploid that harbors similar physiological advantages and a greater genetic diversity.

This study posed for a great insight into the potential of diversity in ploidy in *Sphagnum* from different places of origin. BeadaMoss hold many other species with varying origins which could possess individuals considered to be haploid that are in fact diploid or potentially polypoidal. If the methodology development did not take up as much time of the project as it did then it would have been a possibility to expand the investigation to a multitude of species. The incorporation of GIS software to create a visual map representation of the ploidy levels in micropropagated material would have been a strong baseline to develop further building an entire reference archive of all species housed at BeadaMoss. Investigating spatial genetic structures have often been found to be a successful process and could provide complimentary data when investigating ploidy also. The use of microsatellite loci, a method in which short segments of DNA are amplified can highlight phylogenetic relationships of populations (Vera et al., 2016). A method which has been seen to be highly effective at distinguishing regional differences in *S. magellanicum* between the Beringian and the Atlantic (Kyrkjeeide et al., 2016). Carrying out research into this may help provide insight into why certain locations across Europe have higher levels of ploidy, whilst helping to gain insight into places of origin in which could host the required conditions for a whole genome duplication from diploid *Sphagnum.*

Conclusion

This study set out to better understand ploidy on a regional scale. Attempting to outline potential locations in which various *Sphagnum* species could be identified with higher levels of ploidy. This study highlights an increase in diploid genomic content in *S. fallax* from a German origin which does support the idea of regional ploidy diversity in bryophytes and the *Sphagnum* L genus. The findings acquired support academic literature in the argument of *Sphagnum* being in fixed heterozygosity in non-hybridized specimens, resulting in unequivocally haploid (n=19 chromosomes) or diploid (n=38 chromosomes) species in naturally occurring populations. Potential of whole genome doubling (WGD) has been outlined to be prevalent in ancient populations of *Sphagnum* and could very well occur again, but to what functional processes required and timescales are uncertain. The study into allopolyploid *Sphagnum* could aid in the understanding of spatial ploidy increases and would provide useful samples for archiving and comparison against micropropagated material. Limitations occurred in this research predominately due to time constraints, resulting in the number of species of origins substantially reduced. Bryophyte and *Sphagnum* a like pose for extremely rare instances of WGD unlike angiosperms. The ability to assess a wider set of locations in Europe from a wider number of species could have possibly uncovered other species with higher levels of ploidy occurring naturally. Progression of studies such as this are poised for exciting outcomes, ongoing research is plenty in spatial distribution of genetic compositions in plants and *Sphagnum* a like.

The application of genetic sequencing techniques, continual flow cytometry, and archiving could potentially create a better understanding of regional ploidy diversity in *Sphagnum.*

Executive/ Business Summary

This summary is written for BeadaMoss Micropropagation Services to provide precise relevant information where context is already understood.

Executive/ Business Summary

1.) **Competent nuclei extraction process has been achieved alongside a functional method to analyse *Sphagnum* material in a high throughput format. –** Throughout the methodological development testing of several buffers, fluorochromes and, chopping practices have been synthesised into a simple nuclei extraction process that can be followed for future extraction of *Sphagnum* based material. Following the protocol should enable a high throughput quick extraction that will result in a stable suspension of nuclei with low amounts of debris. Alongside a functional extraction protocol, a second protocol is offered outlining set up and calibration of BD flow cytometers to ascertain credible results. A final protocol is also outlined discussing acquisition, strategic gating, recording and analysing flow cytometric data in *Sphagnum* specimens. The three protocols together provide a fully comprehensive method to analyse *Sphagnum* from micropropagated material. Improvements to elements of the method could still be refined in terms of harnessing greater nuclei counts by the potential usage of higher amounts of LB01 buffer in diploid samples.

2.) **Ploidy levels of Micropropagated material are better understood, highlighting similar ploidy and DNA estimations as references standards. –** Micropropagated material from the *Sphagnum L* genus displayed ploidy characteristics like that of academic literature. Estimated DNA amounts matched values outlined in the Kew c-value standards. Nine species of micropropagated *Sphagnum* now have known ploidy values and more species could easily be quantified. This suggests firstly that the species used in the study are in fact the ones thought, making a useful tool for identification of species that appear physiologically similar. Secondly this would implicate that there isn't any large-scale change in ploidy level when using a micropropation process in comparison to natural populations. Standards have been outlined in haploid and diploid species which can be used as a reference when analysing *Sphagnum* material for future studies. One species standard which did not provide a reflective result. *S. denticulatum* provided a haploid reading but not of the correct estimated DNA size (recording- 0.61pg) standard 0.39pg). This can be implicit of either improper instrument alignment or more likely due to all other species providing credible results a sample that could be a different species. The only reference with a value within (0.05pg) is *Sphagnum recurvum* (0.56p). Improvements could have been made by gaining a species/ taxonomically similar sample with a polypoidal nature to provide an entire outlook on how ploidy is represented using flow cytometry.

3.) **A wide screening of oryzalin induced material at various thresholds of exposure outlined an optimum concentration of 120um but did not create entirely polypoidal specimens.** Concentrations of higher oryzalin (120um) displayed characteristics initiating higher variance in relative fluorescence when compared to a non-induced standard. Higher levels of provide a greater potential of a whole genome duplication. Although polyploidy was not present in samples analysed a large amount of variance was seen in some diploid species. This could suggest that oryzalin is inducing a mixoploid state in *Sphagnum* specimens creating a mosaicism of two genetically different cells. Through the means of subculturing and possibly secondary oryzalin inoculation an entire genome duplication could be achieved. Although attempting to induce polyploidy was not as successful as first hoped this study has identified the first ever increase in ploidy level using oryzalin in *Sphagnum* from *S. squarrosum* creating a diploid variant. On a whole *Sphagnum* did display positive responses to oryzalin providing novel samples of interest and has a great deal of potential in future inoculation trials with the possibility of cell sorting.

4.) **Species of origin displayed regional genetic differences in haploid species however supported the idea of fixed heterozygosity in diploid specimens.** Specific regional genetic novelty in *Sphagnum* was only apparent in one case in a diploid individual of *S. fallax* from a German location. Other haploid species displayed minimal differences from a control sample, but the lack of diversity c could be simply explained by the small number of species analysed. The findings acquired support academic literature in the argument of *Sphagnum* being in fixed heterozygosity in non-hybridized specimens, resulting in unequivocally haploid (n=19 chromosomes) or diploid (n=38 chromosomes) species in naturally occurring populations. It wouldn't appear that *Sphagnum* diverged before the prevalence of polyploidy evolutionary speaking as ancient WGD events have occurred multiple times throughout *Sphagnums* timeline, trying to predict how and when this could occur is difficult to speculate due to a multitude of biotic and abiotic processes. The investigation of allopolyploid specimens described in the literature could expand the understanding of polyploidy relationships in *Sphagnum* and be useful to examine to archive. The interactions of hybridized polyploidy *Sphagnum* in carbon farming and peatland rejuvenation would be useful to examine. Applying accompanying genetic sequencing techniques, flow cytometry and, archiving could provide a solution to the underlying causes of regional ploidy novelty in *Sphagnum* species.

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