The in vitro Culture and Regeneration of Elite Canadian Barley Genotypes

BY

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A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
for the Degree of

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1.0 Abstract

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In order to use tissue culture to improve elite Canadian genotypes of barley (Hordeum vulgare L.), current tissue culture protocols require revision. Previous studies in barley immature embryo culture have determined that both callus induction and subsequent green plant regeneration from calli are dependent on the genotype of the donor plant. Media composition, explant preparation, culture conditions and the physiological status of donor plants have also been reported to affect the efficiency of barley embryo culture.

Three studies were conducted to evaluate the effect of genotype, the composition of the initiation media, effect of explant preparation, the composition of the regeneration media and the location in which the donor plants were grown on the success of barley embryo culture. "AC Bacon", "AC Metcalfe" and "Oxbow" were the elite Canadian cultivars evaluated in the field and growth cabinet studies, and the cultivar "Golden Promise" was also included as it is regarded as the model cultivar in barley tissue culture systems. The greenhouse study (Section 4.0) evaluated the same cultivars except "AC Metcalfe" was replaced by "AC Rosser". Immature zygotic embryos were used as the explant tissue for all studies. Explant preparation was evaluated by comparing intact immature embryos to microsections of immature embryos as the initial explant. Immature embryos collected

from donor plants grown in the greenhouse, field plot or growth cabinet were compared as explants. Callus initiation media and regeneration media were all modified types of Murashige and Skoog (MS) media (Murashige and Skoog,1962).

The ability of explants to generate embryogenic callus was dependent on the genotype of the donor plant. The ability of that embryogenic callus to subsequently regenerate green plants was also dependent on genotype. The genotypes and treatments that produced the highest frequencies of embryogenic calli were not necessarily those that regenerated the highest frequencies of green plants. Plants regenerated from callus were grown to maturity and were normal and comparable in appearance to control plants grown from seed. Very few albino plants were observed (only one plant from Section 5.0). This was unusual as barley tissue culture is frequently reported to yield large numbers of albino plants. Embryogenic calli from the cultivar "Oxbow" were the most regenerative (30% of the embryogenic calli regenerated plants) when the donor plants were grown in the field plot (Section 5.0) and embryogenic calli from the "Golden Promise"cultivar were the most regenerative calli where donor plants were grown in the growth cabinet. Although "AC Metcalfe" explants produced 100% embryogenic calli when donor plants were grown in the growth cabinet, only 8% of those calli regenerated plants. "Golden Promise" embryos yielded the highest frequencies of embryogenic callus when donor plants were grown in the greenhouse (double the percentage generated by the other three cultivars tested). This supports the use of "Golden Promise" as a model barley cultivar for tissue culture. "AC Metcalfe" embryos produced large numbers of embryogenic calli

when donor plants were grown either in the field or growth cabinet, but many of those calli did not give rise to plants. Therefore callus produced from "AC Metcalfe" embryos was less regenerable than callus arising from "Oxbow" embryos.

2.0 Introduction

Barley (Hordeum vulgare L.) has been a staple food for over 18,000 years (Wendorf et al.1979), being the main food cereal of the Roman Empire and referred to in a bible story in which 5,000 people were fed by "five barley loaves and two small fishes" (St. John's Gospel, ca. 90AD). Excavated sites near Aswan in southern Egypt contained plant remains including barley seeds from the late Paleolithic era around 18,000 years ago (Wendorf et al,1979). Barley lost popularity in 16th century Europe when it became regarded as food for the lower classes. Later, wheat superceded barley as a food cereal and today barley is used mainly for animal feed, malt, beer and whisky.

Barley is the second largest cereal crop produced in Canada. Between 1988 and 1997 the average acreage sown to barley in western Canada was 4.2 million hectares, yielding, on average, 11.4 million tonnes of grain annually (Statistics Canada, 1997). Malting barley cultivars account for 70% of Canadian production while feed barley cultivars comprise the remaining 30% (Edney and Tipples, 1997). Malting cultivars can be sold at a premium to the malt and brewing industry if the quality is suitable, with the remainder sold as livestock feed. It is estimated that approximately 65% of western Canadian barley production is used by the Canadian livestock industry (Scott, 2000).

Plant tissue culture and plant transformation technologies are poised to play a key role in improving the barley crops of the future. Plant transformation will allow for the

introduction of specific genes to the barley crop to improve both its agronomic performance and its quality attributes. It will allow for the creation of tailor-made cultivars to meet specific end uses and to fill emerging niche markets.

Canada is the world's second largest exporter of malting barley with 34% of the market share (de Kemp, 1999). The United Sates is the largest importer of Canadian 6-row malting barley. Canadian barley is desirable due to the increasing levels of Fusarium head blight infestation affecting the U.S. barley crop (Cuthbert, 1999). Therefore improving disease resistance in elite Canadian barley cultivars is very important and plant transformation offers a new way to achieve this goal.

Quality improvements, particularly to fill niche markets, will help Canada to maintain or increase its share of the global barley market. It is believed that the main factor in determining Canada's future success in global barley trading will be the development of new cultivars that can meet customer specifications and enable Canada to compete successfully against Australian and European cultivars (Cuthbert, 1999).

Oats and barley are the cereals richest in soluble dietary fibre due to their mixed linkage beta-glucans. Dieticians and nutritionists agree that a food high in soluble fibre, such as oats or barley, can help to lower blood serum cholesterol levels. However considerable quantities must be consumed in order to achieve these benefits (Salge Blake, 1997).

Plant transformation may allow researchers to raise the level of these beneficial beta-glucans in barley, creating a niche market for barley as a nutraceutical or functional food.

Specific quality attributes are sought by different end users, i.e. the feed industry, brewers and maltsters or human food markets. Genetic engineering may eventually allow for tailor-made barley cultivars to meet each demand. The feed industry is interested in hulless barley (Bhatty, 1996) since a reduced hull content increases the digestible energy of the grain (to obtain more pork and less manure). Hulless barley production has grown over recent years and producers are also becoming interested in the use of barley for forage (Therrien, pers comm., 2000). Barley is a constituent of hog and poultry feeds, among others, where it is used mainly as an energy source for the livestock. However the protein content and quality of the grain give barley an advantage over wheat or corn. Cultivar differences of up to 40% with regard to voluntary intake of barley feed by poultry and pigs have been identified by the Prairie Swine Centre (Scott, pers comm., 2000) although the factor(s) responsible for these differences have yet to be identified.

Barley may also be useful in the feed industry if it could be improved as a functional feed. In particular, having barley produce natural antibiotics would remove the need to use synthetic antibiotics which have been banned in Europe and remain under pressure in other countries. Genetic engineering could be a way to achieve this goal.

The malting industry pays a premium for barley that meets their standards for quality. Canada's largest market for malting barley is the domestic malting industry. One of the maltsters' main concerns is the extent of hull peeling. The hull should be strong enough to remain relatively intact through malting, transportation and lautering, but thin enough to minimize wort polyphenol levels. In contrast to the dieticians and nutritionists, brewers and maltsters want low beta-glucan and polysaccharide levels in their barley to minimize wort and beer viscosities for easier filtration and to reduce potential haze problems in the beer. The beta-glucans form very viscous solutions causing problems with filtration and beer storage. The desired reduction in beta-glucans could be accomplished either by reducing the beta-glucan content in the grain of malting cultivars, or alternatively, by increasing the beta-glucanase content (beta-glucanase enzymes being responsible for the degradation of beta-glucans). It might be possible to achieve this by replacing the original beta-glucanase promoter with one that is more transcriptionally active to increase expression of the glucanse gene.

Increased carbohydrate content (fermentable extract) would be a benefit to the malting industry, but is unlikely to be achieved using the present range of germplasm available where only small increments might be achieved through conventional breeding techniques. Malting barley should have as high a starch content as possible. During brewing, starch is converted to fermentable carbohydrate by enzymes in the malt. Some desired starch characteristics include a low gelatinisation temperature and rapid conversion of starch to fermentable sugars (MacGregor, 1999). An increase in

carbohydrate levels might be achieved through the application of tissue culture and transformation methods.

Malting barley should contain consistent grain protein levels (between 10-12%) even if the plant was exposed to stressful growing periods. The protein content should be high enough to provide sufficient nutrition for fermentation and peptides for beer head retention (Yin and Fernets, 1999). Brewers are also looking for low DMS-p (di-methylsulphide precursor) levels. The DMS that is produced from precursors in the barley gives beer its characteristic flavour when present in low concentrations, however higher DMS-p levels are not desirable. Brewers are also looking for lower linolenic acid levels to minimize the tendency of beer to stale.

When developing food markets for Canadian barley one must consider that establishing a new market is both complicated and expensive. One of the more obvious markets for barley as a food is Japan (Klafke, 1999). The Japanese have a long history for using barley as a foodstuff. It is used as a rice extender, for tea, miso soup and for shochu (liquor). Since all of these products are very different, the specifications for the raw barley used in each are also different. To be able to capitalise on these markets, Canada must be able to supply cultivars that meet the customers' specifications and this, in turn, requires Canadian cultivars specifically designed to meet these quality attributes. Tissue culture and other biotechnological techniques such as transformation may provide a means by which these cultivars may be produced within a much shorter time period than

that required for traditional breeding methods. In addition, these procedures can be used to produce varieties with a range of traits not achievable by conventional breeding methodologies. Target traits could be improved agronomic properties, disease resistance, specific quality attributes or improved nutritional value.

It is apparent that the different markets for barley each have certain specific criteria that the cultivars must meet to be considered candidates for use in those markets. One way by which existing cultivars may be improved to better meet those criteria is through biotechnology. An efficient culture and regeneration system is a prerequisite for barley improvement through biotechnology.

This research will determine optimal parameters for barley tissue culture and regeneration and as such, will provide a foundation for future studies in tissue culture and barley transformation. In particular, these studies will determine the optimal explant treatment and regeneration medium for each of the cultivars studied using zygotic embryos derived from donor plants grown under different conditions (greenhouse, field and growth cabinet). Parameters examined include the physiological status of the donor material as determined by the growth conditions, explant preparation, media composition and genotype-specific responses. Previous studies have indicated cultivar-specific responses to barley tissue culture (Dale and Deambrogio, 1979; Goldstein and Kronstad, 1986; Lührs and Lörz, 1987; Hanzel et al , 1985; Bregitzer, 1992). In addition, explant preparation has been examined in previous studies (Wernike and Milkovits, 1987a,b;

Cheng and Smith, 1975; Weigel and Hughes, 1985; Saalbach and Koblitz, 1978). A recent study by Dahleen (1999) examined the growth conditions of donor plants and Bregitzer et al (1999) evaluated specific media preparation methods. None of these studies have evaluated cultures of the elite Canadian lines examined here.

The specific objectives of this research are:

1) to evaluate the initiation media for establishing barley cultures from immature zygotic embryos;

2) to evaluate efficiency of those cultivars in terms of explant ability to
   a) generate embryogenic calli;
   b) regenerate intact plants from this callus;

3) to examine the effects (if any) of different regeneration media on regeneration of green plants from calli;

4) to compare the growth location effect (if any) on the donor material using plants grown under greenhouse, field plot and growth cabinet conditions;

5) to compare the treatments of immature zygotic embryos in preparing explants for culture. Explant preparations were either intact embryos or micro-sectioned embryos.

By examining these parameters, we hope to establish an efficient culture and regeneration system for elite Canadian barley cultivars, thereby facilitating their use in future barley transformation studies. It is hoped that culture efficiency will be comparable to, or better than that of the "Golden Promise" model cultivar.

3.0 Review of the Literature

3.1 Tissue Culture

Tissue culture is the propagation of cells or tissues within a sterile environment. Usually the cells or tissues cultured (referred to as explants) are removed from donor plants, sterilised as appropriate and then initiated into culture by placing on or in, a culture initiation medium. Tissue culture protocols are usually species-specific although there are common features such as the requirements for sterility, appropriate nutritive media and a totipotent explant for regeneration.

In order to achieve specific goals which cannot be obtained through conventional breeding, such as the introduction of a gene from another species, one must have an efficient method of recovering fertile plants from cultured cells. For the regeneration of fertile plants from culture, a totipotent cell line is required. Totipotent means that the cell or cell line may be taken at any stage in the differentiated developmental process yet retain the potential to produce de-differentiated "callus" cells under specific cell culture conditions and subsequently, re-differentiate to produce whole plants. An example of totipotent cells may be taken from a study on sugar beet (Hall et al, 1996), where plants were regenerated from callus of stomatal guards cells. However, there is debate over whether all plant cells are actually totipotent. It has been suggested that some have limited totipotency (unipotent) and some actually lose this ability completely (nullipotent)

making those cells unable to produce callus and regenerate plants (Tran Thanh Van, 1981). While the exact pathway followed in the regeneration of plants through de novo synthesis by organogenesis or embryogenesis is unclear, it is known that the explant cells must be competent. In the context of tissue culture studies, the term competence is used to refer to totipotent cells that are able to identify and respond to inductive signals such as plant growth regulators. Those cells then become committed to a particular developmental pathway.

Somatic embryogenesis can be defined as the formation of an embryo from a cell other than a gamete or the product of gametic fusion and is a result of the totipotency of plant cells. To reprogram a cell to an embryonic state, current gene expression must be terminated and replaced with expression of embryonic genes. One study correlated the down regulation of gene expression through DNA methylation with the concentration of exogenous auxin applied to the tissue (LoSchiavo et al, 1989). Individual genotypes may have specific requirements that determine optimal regenerative capacity. Also a factor in somatic embryogenesis in tissue culture is the probability that the entire embryo may not have embryogenic capacity. For instance, in pea, only the axis is responsive (Kysely and Jacobsen, 1990). Therefore, careful consideration must be given to the choice of explant tissue.

Tissue culture and related plant cell culture manipulations are necessary for many techniques used in crop improvement. For example, tissue culture plays a major role in

the production of haploids and doubled-haploids (anther or microspore cultures), embryo rescue (from developing seeds), somaclonal variation, plant transformation, for production of somatic hybrids and also for selection of mutants by in vitro selection. This is possible, because under in vitro conditions it is possible to identify certain cells within a large population for tolerance or resistance to biotic and abiotic stresses such as herbicides, pathogens or toxins, high salt concentrations, heat or cold stresses. For instance, the pathotoxin, fusaric acid, of Fusarium was used by Chawla and Wenzel (1987) to successfully select callus derived from immature embryos of barley for tolerance and resistance. Similarly, anther-derived embryogenic calli were used to select for improved frost-hardiness in barley (Tantau et al, 1995) although it has yet to be proven whether the selected characters are stably transmitted to progeny of the primary regenerants.

While some success has been achieved in the tissue culture and subsequent regeneration of barley, most of this has been with the "Golden Promise" spring cultivar or other cultivars with little or no agricultural importance. In order for tissue culture to contribute to crop improvement, efficient regeneration of fertile plants from culture is a prerequisite. In Canada in particular, in order to efficiently integrate gene transfer technology into a barley breeding program, it would be advantageous to directly transform existing elite Canadian lines. Transforming elite lines allows one to produce a desirable plant in a much shorter time than if transformation was carried out on unadapted genotypes. Although the introduction of foreign genes into unadapted model genotypes is possible,

one must take into consideration the possible occurrence of deleterious effects such as transgene silencing, differences in transgene expression in different genotypes, and the effect of linkage drag where the transgene is integrated in or around undesirable genes in the resulting transgenic plant. The use of elite lines also facilitates the evaluation of the transgene in an appropriate genetic background. For example, where a transformation was carried out to improve an aspect of a malting barley, it makes more sense to initially transform a malting variety to allow the effects of the transformation to be determined appropriately.

3.2 Somaclonal Variation

One cannot ignore the effects of the tissue culture process itself. Tissue culture is understood to promote phenotypic and genotypic variation in the subject cultures, a point illustrated by the spontaneous generation of albino plants from cultures (Bregitzer, 1992, Dahleen, 1999; Bregitzer et al, 1999; Wan and Lemaux, 1994). This phenomenon, where plants grown in vitro are labile, showing various degrees of instability, is referred to as somaclonal variation. With reference to cereals in particular, it can result in regenerated plants (transformed or non-transformed) which are stunted, have reduced yield and quality of yield, reduced quality of malt and do not form heads properly. Heads can be small, malformed and / or sterile (Jähne-Gärtner and Lörz, 1996). A study on the malting quality of cultured barley genotypes demonstrated that malting quality can be

adversely affected by the tissue culture process (Bregitzer et al, 1995). The generation of albino plantlets also occurs through tissue culture as a form of somaclonal variation where the plantlet does not achieve full photoautotrophic competence. Epigenetic variation is variation in the characteristics of a plant which is not due to changes in the primary sequence of nucleotides. For example, the activation or inactivation of genes by methylation of the DNA is an epigenetic change. Somaclonal variation however, is genetic variation; heritable variation which is due to primary changes in the nucleotide sequence. Genetic variation may arise through chromosomal mutation by changes in ploidy level, point mutations, deletions or translocations. The incidence of somaclonal variation is increased with time in culture and can add to the negative effects of transformation mentioned above. This genetic variation can be observed at the highest frequencies in de-differentiated cultures (callus cultures) and tend to occur soon after the culture is initiated. Not all characters change through this variation and the changes can be negative. These changes are generally unstable and are not expressed in the whole regenerated plant. However, for all its problems somaclonal variation is not without its advantages in plant breeding. Changes can occur in useful agronomic characters and these changes can occur at extremely high frequency. This is also a means by which novel changes can be achieved without the use of genetic transformation. Somaclonal variation effects can detract from the benefits of a successful transformation event. Although the regeneration of plants through culture is asexual, the resulting plants can still be affected by somaclonal variation.

3.3 Initiation of a Tissue Culture

3.3.1 Physiological Status of Donor Material

The growth environment of the donor plant strongly influences the quality of explant obtained from that material and thus, affects the quality and efficiency of the culture. Plants which are grown in stressful environments cannot be relied upon to produce high quality explants for culture. Appropriate steps should be taken to minimise the stress imposed on the potential donor material. Growth conditions can affect different barley cultivars in different ways and to varying degrees (Lührs and Lörz, 1987). An evaluation of greenhouse and growth cabinet environments for growth of barley donor plants indicated that genotype, growth environment and planting date all had significant effects on green plant regeneration levels (Dahleen, 1999).

3.3.2 Choice of Explant

In cereal tissue cultures, many parameters must be considered to ensure the success of the culture. Of these parameters, the choice of explant to establish the culture is particularly important. This choice is governed by the particular species and genotype to be cultured and by the type of culture one hopes to establish, for instance a suspension culture or embryogenic culture. Certain explants are better for some types of culture than others. Studies by Dale and Deambrogio (1979), Hanzel et al, (1985) and Goldstein and Kronstrad (1986) have recognised that the regeneration of barley through in vitro methods is extremely genotype-dependent. In addition, a study by Lührs and Lörz

(1987), which examined 41 barley cultivars for efficiency of callus formation, determined that somatic embryo formation by the induced callus is also influenced by genotype.

Initiation of a barley tissue culture requires an explant of immature meristematic tissue since it has proven extremely difficult to induce cell division and de-differentiation in more mature tissues (Wernike and Milkovits, 1987 a,b). Also, immature material tends to have a higher degree of competence than mature tissues. A variety of explants have been used in barley tissue culture for protoplast, callus and cell suspension cultures. Seedlings were used as donor material for apical meristem explants (Cheng and Smith, 1975). Cheng and Smith induced callus within 3-5 weeks by culturing their barley meristems on MS medium (Murashige and Skoog, 1962) supplemented with auxins. Regeneration was achieved through organogenesis by transferring the callus pieces to a regeneration medium (MS medium lacking auxins). Weigel and Hughes (1985) also regenerated plants from somatic embryos using barley meristem explants. When using seedlings as donor material, it is important to consider the degree of morphogenic competence within the seedling as in barley, there appears to be a difference between the percentage of callus induced from the leaf base and the mesocotyl (Jelaska et al, 1984). Sections closest to the seedling axis had higher callus formation frequencies than more distant ones (Becher et al,1992). Saalbach and Koblitz (1978) reported that regeneration of plants from differentiated leaf segments was very difficult. While callus formation was achieved, regeneration from the callus pieces was rare. Mesocotyl-derived callus

has been used to establish cell suspension cultures of barley but these particular cultures were found to be non-morphogenic (Müller et al, 1989).

In barley cultures, plant regeneration through embryogenesis is easier to achieve using immature zygotic embryos as an explant to establish a culture. The enhanced regeneration system developed by Weir et al (1996a,b), is based on establishing cultures of isolated scutella from immature zygotic embryos. Research from various barley studies have demonstrated that immature zygotic embryos are the most favorable explant for initiating an embryogenic culture (Dale and Deambrogio, 1979; Hanzel et al, 1985; Thomas and Scott, 1985; Goldstein and Kronstrad, 1986; Lührs and Lörz, 1987; Lazzeri and Lörz, 1990; Huang et al, 1993; Ritala et al, 1994; Wan and Lemaux, 1994; Hagio et al, 1995). The resulting callus is generated by the peripheral cells in specific regions of the embryonic scutellum (Lu and Vasil, 1985; Vasil, 1987). Callus from immature embryos tends to be compact and nodular with a yellow colour (Dale and Deambrogio, 1979). This type of callus is embryogenic since the nodular aspect is in fact, somatic embryo formation. The other type of callus is referred to as non-embryogenic and is characterised by watery, white cells. Even though in previous studies, it is characterised as non-embryogenic it is possible that plants can still be regenerated from it. Perhaps a more accurate description would be poorly embryogenic rather than non-embryogenic.

The orientation of the barley explant on the medium must also be given consideration as some immature embryo explants fare better when placed scutellum side down. Other

explants of the same tissue fare better when placed axis-side down. Some studies have involved initiating the culture with the explant in one orientation then switching it to the other orientation, usually for barley transformation protocols (Tingay et al, 1997).

The age and size of the explant is important and optimal embryo lengths ranging from 0.5 to 2 mm have been suggested by different studies (Goldstein and Kronstrad, 1986; Dale and Deambrogio, 1979; Thomas and Scott, 1985).

In one study (Golds et al, 1993), microsections of immature barley embryos produced considerably more callus and somatic embryos than cultures initiated from intact embryos. They found that coleoptilar sections of the scutellum gave the most callus and were highly embryogenic. The sectioning increases the wounded surface area and appears to enhance the callus response by increasing the surface area for uptake of hormones and nutrients from the medium. Another advantage of the microsection technique in barley is that larger sized embryos (from 2.5 - 3.0 mm) which might otherwise be too mature to be useful in culture initiation can be induced to produce more callus than when cultured as intact embryos. In addition, less precocious germination was observed from microsectioned explants (Golds et al, 1993). Precocious germination occurs when an immature embryo germinates in the culture plate rather than generating callus. This type of germination is frequently observed in monocotyledonous cultures such as barley (La Rue, 1936). For transformation protocols, this wounding technique has also been applied by micro-projectile bombardment of the target tissue prior to

inoculation with Agrobacterium to increase the surface area available for infection by Agrobacterium and to increase transformation efficiency (Bidney et al, 1992).

Immature inflorescensces can be used to establish barley cultures, however, they are not often the explant of choice and therefore, there is little information available (Botti and Vasil, 1984; Thomas and Scott, 1985). When using this type of explant, callus initiation can take around 6 weeks which is twice as long as callus initiation from immature embryos. Inflorescence leaf sheaths have also been used to establish barley cultures and while callus induction was achieved, embryogenesis and organogenesis did not occur (Barcelo et al, 1991).

The culture of unfertilised ovaries gave rise to haploid barley plants through gynogenesis (San Noeum, 1979). The rates of callus induction and plant regeneration were 2 to 3-fold higher from cultures initiated from ovaries cultured from florets with trinucleate pollen compared to cultures initiated from florets with binucleate pollen (Callisto and Cistué, 1993) . Ovule culture studies have demonstrated that unfertilised ova were not regenerable although ova isolated 90 minutes after pollination could produce embryos (Topfer and Steinbiss, 1985).

Anther and microspore culture are useful to establish a barley tissue culture, with the isolation of intact anthers being the most usual explant (Dunwell, 1985; Luckett and Davey, 1992; Pickering and Devaux, 1992). This particular type of culture was initially

researched by Clapham (1971, 1973) and since then, the methods have been optimised to the point where the technique is used routinely by breeders for the production of homozygous plants.

3.3.3 Choice of Culture Medium

Another parameter that determines the efficiency of a tissue culture is the choice of initial medium for callus induction and thereafter, for plant regeneration through organogenesis from the induced callus. Although some progress has been made in the transformation of barley, one particular problem was common to many of these studies: the inability to regenerate fertile plants from the transformed tissues (Kartha et al, 1994). Optimisation of genotype-specific culture protocols concerning such factors as choice of explant, media composition and growth conditions of the donor material, should help to reduce this problem.

Different induction media have been successfully used to induce callus in many barley studies, thus the composition of basal salts does not appear to have a dramatic influence on this aspect of the culture process. The most well documented basal medium for callus induction is the MS medium (Murashige and Skoog, 1962) and this has been used by many researchers (Hanzel et al, 1985; Thomas and Scott, 1985; Breimann, 1985; Karp and Lazzeri, 1987; Lührs and Lörz, 1987; Wan and Lemaux, 1994; Hagio et al, 1995). However, Gamborg et al (1968) developed B5 medium which has also been frequently used (Dale and Deambrogio, 1979; Breimann, 1985; Goldstein and Kronstrad, 1986;

Lührs and Lörz, 1987; Ziauddin and Kasha, 1990). In many cases, the basic media types have been supplemented with various additional components such as increased thiamine and / or inositol concentrations (Cheng and Smith, 1975; Weigel and Hughes, 1985; Lührs and Lörz, 1987) and varying concentrations of plant growth regulators such as auxins (Skoog and Miller, 1957; Dale and Deambrogio, 1979; Hanzel et al, 1985; Goldstein and Kronstrad, 1986; Lührs and Lörz, 1987). Usually 2,4-dichlorophenoxy acetic acid (2, 4-D) or one of its analogues, are the preferred auxins for embryogenic callus induction with optimal concentrations varying from 1-5 mg/l (Golds et al, 1993). Another supplement to the basal media is increased concentrations of copper sulphate (Dahleen, 1995). The FHG medium contains less ammonium nitrate and increased glutamine (Gustafson, 1990). Other supplements may be coconut water or casein hydrolysate. Also, the choice of carbon source is important as it too is culture specific. Initially, sucrose was used but since Hunter (1988) demonstrated the superiority of maltose over sucrose in barley microspore cultures, the majority of studies now use maltose. These supplements or variations may be made to the callus initiation media and / or to the plant regeneration media and are also determined by the explant used to establish the culture and the type of culture to be initiated. The actual preparation of the media might also have an effect on the efficiency and quality of the culture. Bregitzer et al (1998) suggest preparing and autoclaving certain components separately to avoid precipitation of certain components of the barley media.

3.4 Transformation Studies

The callus derived from immature embryo culture and the immature embryos themselves have been used successfully in barley transformation protocols using particle bombardment or Agrobacterium (Ritala et al, 1994; Wan and Lemaux. 1994; Hagio et al, 1995, Tingay et al, 1997). The resulting transgenic barley plants were not chimeric which points to an earlier suggestion that somatic embryos are of unicellular origin.

4.0 Barley Immature Embryo Culture Using Greenhouse-Grown Donor Plants

4.1 Introduction

In my first study, I cultured immature zygotic embryos derived from donor plants grown in a greenhouse environment to evaluate several parameters which are known from previous studies to have a bearing on the efficiency of barley tissue culture. The following hypotheses were tested:

1. Cultivar has a significant effect on the efficiency of embryogenic callus formation and subsequent regeneration of plants in a barley culture system

2. Composition of the initiation medium and / or regeneration medium will affect the efficiency of embryogenic callus formation and subsequent regeneration of plants in a barley culture system

In order to test these hypotheses, donor plants of four cultivars were grown in the greenhouse to provide explants for culture initiation. Two types of initiation media and three types of regeneration media were tested. The study was conducted in two phases.

Phase one was the culture initiation and callus generation phase and phase two was the regeneration of green plants from the induced callus.

4.2 Materials and Methods

4.2.1 Growth of Donor Plants and Harvest of Spikes

Plants of three Canadian barley cultivars ("Oxbow", AC Rosser" and "AC Bacon") and "Golden Promise" were grown from seed under greenhouse conditions in accordance with a standard protocol (Watson, pers comm., 1998). Daylight was supplemented with metal-halide lamps at 230 μE $m^2 s^{-1}$. Temperatures fluctuated widely between 20°C and 30+°C. Breeder's seed was planted in soil-filled clay pots of 15 cm diameter and fertilised on seeding and once per week for the first three weeks with Plant-Prod® water soluble fertiliser (20% nitrogen, 20% phosphorous and 20% potassium). 5g of fertiliser was applied to the soil surface of each pot and then watered in (the exact volume of water applied to each pot was not measured). Plants were watered every 3 days. The following weeks, Border Brand fertiliser with 11% nitrogen and 52% phosphorous was used to fertilise once per week. This fertiliser was applied in the same manner as the Plant-Prod®. After approximately 14 days post anthesis (Bregitzer et al, 1992), spikes were examined daily for caryopses at the required stage. This is primarily a visual and physical test where a seed would be selected from around the centre of a potential spike. By touching the seed, one can tell (with practice) whether the embryo would be at the

necessary stage of development. By squeezing the seed over a fingernail, the embryo can be pushed onto the fingernail and observed. If no embryo is present, or the endosperm is watery, the spike is not harvested. If the embryo is small but still visible and identifiable as an embryo with more doughy endosperm, the spike is harvested. Healthy spikes at this stage were cut from the plants and, where the material could not be cultured immediately, stored in sealed plastic bags at 4°C for a maximum of 7 days. This is a modification to a wheat culture protocol which stored spikes for up to 5 days in water at 1°C (Altpeter et al, 1996). Spikes were stored at 4°C to minimise further maturation of the embryos and to preserve the plant material (Weir et al, 1998).

4.2.2 Embryo Culture

Prior to culturing, media was prepared and equipment was autoclaved. Two different kinds of initiation media were used in this study. The first was a modified MS (Murashige and Skoog, 1962) medium, and the other was the MS medium supplemented with $CuSO_4$ (Dahleen, 1995). Three types of regeneration media were tested - regular regeneration medium, regular plus $CuSO_4$ (Dahleen, 1995) and FHG (Gustafson, 1990). Appendix 1 details the components of each of these media types and how each was prepared.

Caryopses were removed from the cut spikes and the hulls were peeled off. As each caryopsis was peeled, it was placed in a petri dish containing 15ml of distilled water to prevent dessication. After all caryopses were peeled, the water was drained off and the

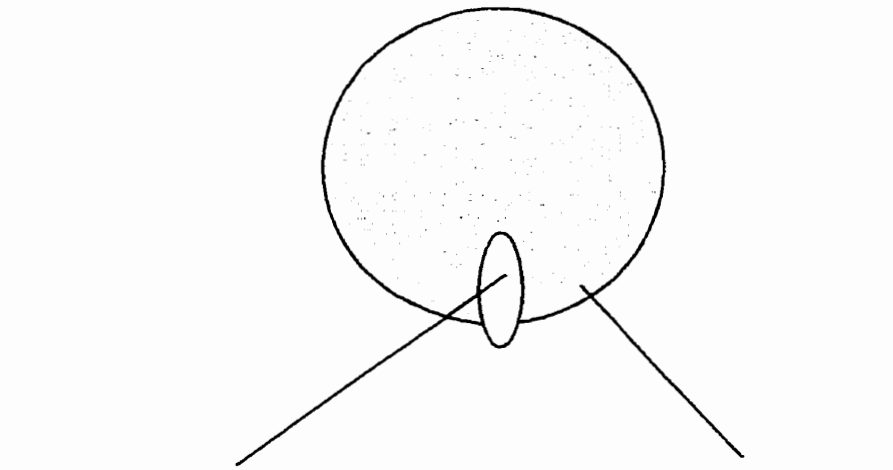
caryopses were scooped into a wire mesh tea-ball to permit easy transfer between sterilising solutions. The caryopses were then surface sterilised (Kartha et al, 1994). The tea-ball with the caryopses was immersed in 100 ml of 70% ethanol for 1 minute with agitation. The tea-ball was then removed and tapped on the side of the beaker to shake off excess ethanol before being placed in 100 mls of a 20% (v/v) Javex® solution (Javex® is 1.2% sodium hypochlorite) for 20 minutes. The solution containing the caryopses was placed on a shaker at medium speed to ensure thorough contact with the Javex®.

While the caryopses were in the Javex® solution, the laminar flow cabinet was prepared by swabbing all surfaces with 70% ethanol and swabbing all necessary equipment with 70% ethanol before placing them in the cabinet, including the stereo dissecting microscope.

After the 20 minutes had elapsed, the tea-ball containing the caryopses was removed from the Javex® solution, placed in a sterile beaker and rinsed 3 times with sterile, distilled water (Dahleen, 1995). Gloves were not worn but hands were washed with soap and water before entering the cabinet. Great care was taken to maintain asepsis at all times. As a result, microbial contamination was rarely a problem. Other than during surface sterilisation, no antimicrobial agents were applied.

One by one, each caryopsis was selected and using two pairs of sterile, fine point forceps, the embryo was "popped" out of the base of the seed by applying pressure above the area occupied by the embryo. Forceps were dipped and flamed prior to each time contact was made with an embryo or caryopsis. Embryos were randomly assigned to plates of MS media or MS+Cu media with half the embryos plated on each medium. Approximately ten to fifteen explants were plated on each plate and exact numbers recorded. Each plate was considered as one experimental unit and the total number of plates established for each treatment was considered the number of replicates for that treatment. For example, if there were three plates established of intact explants on MS medium, this was regarded as three replicates for that particular treatment. The immature embryo explants used to initiate these cultures were between 1.0 and 2.0 mm in length from the center-most top of the scutellum to the lowest point of the embryonic axis. All explants (immature zygotic embryos) were cultured intact (referred to as "intact" explants). All were cultured scutellum side down on the medium (Wan and Lemaux, 1994; Bregitzer, 1992; Hanzel et al, 1985). Figures 1 illustrates how the explants were prepared for this study.

Figure 1 Intact Barley Immature Embryo Explant



4.2.3 Callus Initiation and Scoring

After the last explant was cultured, the plates were sealed using Parafilm® then placed in the dark at 27°C to encourage callus growth (Bregitzer, 1992; Altpeter et al, 1996;Wan and Lemaux, 1994, Tingay et al, 1997) . After three weeks, callus and / or explants were sub-cultured to fresh media of the same type (Goldstein and Kronstrad, 1986). Where precocious germination had occurred, the shoots and any roots were removed at this time. After a total of six weeks in the dark, the callus was scored as embryogenic or non-

embryogenic and the numbers of each kind were recorded. Embryogenic callus was defined by the presence of nodular somatic embryos on the surface of the callus. It was cream to pale yellow in colour and was compact with a firm, "crisp" texture. Non-embryogenic callus was white to creamy in colour with a watery translucent appearance and loose texture. No nodular embryos were present. After that, the calli from each induction treatment were divided as equally as possible between each of the three types of regeneration media. During sub-culture, the embryogenic calli had a tendency to break up when touched with the forceps or through any kind of impact to the plate. This sometimes resulted in there being more callus pieces than original explants which complicated the data analysis. In some cases, it was not possible to discern which particular explants on each plate had given rise to the extraneous callus pieces. All pieces were transferred to the fresh media. Since explants were of similar sizes, this did not seem to present a major problem except for analysis of the data. It was decided to record percentages of calli pieces from explants and then to determine what percentage was embryogenic and what percentage was non-embryogenic, thus essentially removing the number of explants as a consideration for analysis.

Callus can be defined as a mass of undifferentiated cells. Callus generated from each explant was scored as embryogenic callus or non-embryogenic callus in accordance with the descriptions outlined above. It was expected that the embryogenic callus would give rise to more regenerated plants than the non-embryogenic type due to the presence of somatic embryos on the embryogenic callus which are lacking on the non-embryogenic

type. While generation of embryogenic callus can be observed within three weeks of culture initiation, it was noted that non-embryogenic callus at this point in the study could also become embryogenic within the next three weeks under dark conditions. Figure 2 shows an example of embryogenic callus with visible somatic embryos, while Figure 3 illustrates a piece of callus with both embryogenic and non-embryogenic characteristics.

Figure 2 **Embryogenic Callus of "Golden Promise" Cultivar Cultured from an Intact Explant Derived from a Greenhouse -Grown Donor Plant**

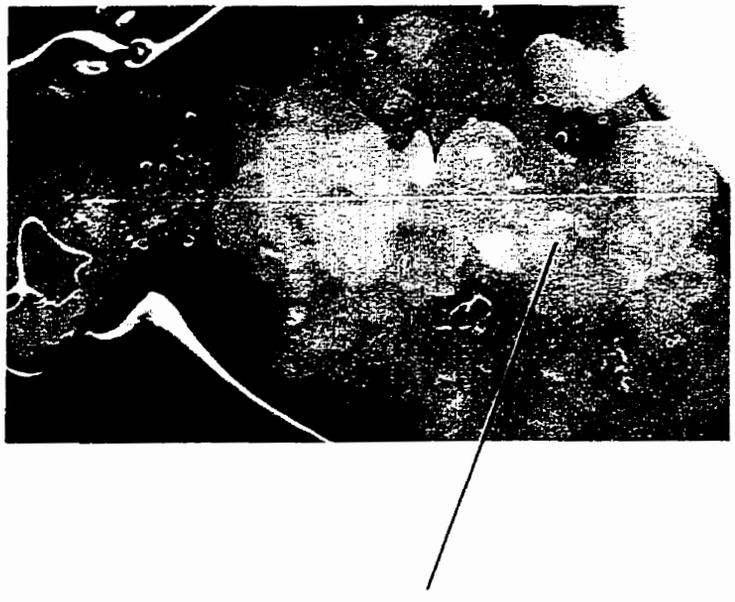
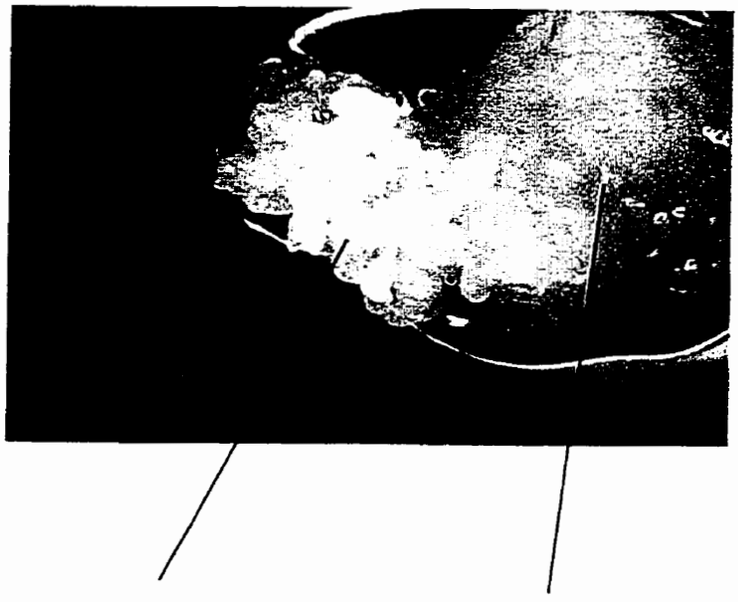


Figure 3 **Embryogenic Callus and Non-Embryogenic Callus of "Golden Promise" Cultivar from Cultured Intact Embryo from Greenhouse-Grown Donor Plants**



4.2.4 Data Collection and Analysis

For each cultivar tested on each type of initiation media, we recorded the total number of explants cultured, the number of replicates (plates), the percentage of explants successfully producing callus and the percentage of these calli which were embryogenic, by replicate.

This was a completely randomised study. The original data (percentage of explants producing callus and percentage of that callus which was embryogenic) did not fit a normal distribution, which is a requirement for either ANOVA (analysis of variance) or GLM (general linear model) tests on the data. We attempted to normalise the data for further data analysis using an arcsine transformation of the square root of the percentage of embryogenic calli generated in the study. Where count data is collected, usually a square root transformation serves to stabilise the variances so that they are not dependent on the mean. However, binary data such as that obtained here (percentages of embryogenic or non-embryogenic calli), tends to have greater variance around 50% and the arcsine transformation equalises the variances more effectively than square root alone, over a range from 0 to 100%.

However, in this case, the transformed data still did not fit a normal distribution, preventing an ANOVA or GLM analysis of the data. Paired T-tests were calculated by

hand on the raw data to test initiation media effects, and cultivar effects. The T-statistic allowed identification of significant and non-significant effects. The formula used to calculate the t-statistic (|t|) is as follows:

$$|t| = \frac{\text{mean of A - mean of B}}{S\bar{d}\ \text{(Standard deviation)}}$$

4.3 Results and Discussion

The average percentage of explants successfully producing callus for each cultivar tested on each type of initiation medium, and the average percentages of those calli which were embryogenic are recorded in Table 1. T- tests were conducted on the raw data within each cultivar to determine whether there were significant differences in formation of embryogenic callus between the two types of initiation media tested (Table 2).

Table 1 **Total Number of Intact Barley Embryo Explants Cultured for Each of Two Media Treatments and the Percentages Generating Embryogenic Calli After Six Weeks**

Cultivar	Initiation Medium	Total Number of Explants Cultured	Number of Replicates (Plates)	Average % of Explants Forming Callus (+/- Std. Err.)	Average % of Calli which were Embryogenic
AC Bacon	MS	62	3	85 ± 8	6 ± 1
AC Bacon	MS+Cu	56	3	86 ± 8	47 ± 5
AC Rosser	MS	45	2	59 ± 12	4 ± 4
AC Rosser	MS+Cu	41	2	63 ± 11	13 ± 13
Golden Promise	MS	50	3	100 ± 0	89 ± 10
Golden Promise	MS+Cu	55	3	75 ± 21	73 ± 23
Oxbow	MS	48	3	66 ± 17	11 ± 6
Oxbow	MS+Cu	45	3	49 ± 25	0 ± 0

Table 2 clearly illustrates that there were no significant differences between initiation media treatments in this study. This would indicate that differences between these particular media do not exert an effect on the formation of callus from immature barley

embryos where the embryos are cultured intact. From this data, it would seem that increased copper sulphate did not enhance the generation of embryogenic calli from explants.

Table 2 **Paired T-Test Results Between Induction Media Types Within Each Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Cultivar	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
AC Bacon (MS vs MS+Cu)	0.11	4	-
AC Rosser (MS vs MS+Cu)	0.52	2	-
Golden Promise (MS vs MS+Cu)	1.5	4	-
Oxbow (MS vs MS+Cu)	0.7	4	-

Table 3 contains the results of the analysis for formation of embryogenic calli from explants on those initiation media. The Table shows that there were no significant differences between the two initiation media in formation of embryogenic calli. Therefore, from Tables 2 and 3, it is clear that in this study, the addition of supplemental copper to the initiation media had no effect on either the formation of total calli

(including both embryogenic and non-embryogenic calli) or the formation of embryogenic calli alone, when tested at the 5% level of significance.

Table 3 Paired T-Test Results Between Induction Media Types Within Each Cultivar in the Formation of Embryogenic Calli

Cultivar	**t-statistic**	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
AC Bacon (MS vs MS+Cu)	2.32	4	-
AC Rosser (MS vs MS+Cu)	0.92	2	-
Golden Promise (MS vs MS+Cu)	0.77	4	-
Oxbow (MS vs MS+Cu)	2.1	4	-

Since there were no significant differences between initiation media, results for each cultivar were pooled over initiation media types. Each pair of cultivars was then tested, using a t-test, to determine whether cultivar had a significant effect on the formation of total calli (including both embryogenic and non-embryogenic calli) formed by the explants. Table 4 shows that cultivar was not a significant factor in determining the total amount of calli generated, at the 5% level of significance.

Table 5 compares the data obtained for each of the four cultivars in the formation of embryogenic calli only, in order to ascertain whether cultivar was significant in determining the quantity of embryogenic calli formed by the intact explants. The table shows that explants of "Golden Promise" were significantly more efficient (at the 1% level) in forming embryogenic calli than "AC Bacon", "AC Rosser" or "Oxbow". The generation of embryogenic calli was the principal aim of this study since embryogenic calli tend to be responsible for the majority of regenerated plants (Dahleen, 1995; Bregitzer, 1992; Bregitzer et al, 1998; Lührs and Lörz, 1987; Goldstein and Kronstrad, 1986; Bregitzer et al, 1999).

Table 4 **Paired T-Test Results Between Cultivars in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Cultivars	|t|	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
Golden Promise vs AC Bacon	0.18	10	-
Golden Promise vs AC Rosser	0.13	8	-
Golden Promise vs Oxbow	1.83	10	-
AC Rosser vs AC Bacon	0.07	8	-
AC Rosser vs Oxbow	1.9	8	-
Oxbow vs AC Bacon	2.02	10	-

Table 5 **Paired T-Test Results Between Cultivars in the Formation of Embryogenic Calli**

Cultivars	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
Golden Promise vs AC Bacon	6.2	10	**
Golden Promise vs AC Rosser	5.98	8	**
Golden Promise vs Oxbow	6.61	10	**
AC Rosser vs AC Bacon	0.4	8	-
AC Rosser vs Oxbow	0.5	8	-
Oxbow vs AC Bacon	1.2	10	-

Unfortunately, the quality of the donor plants in this study was poor due to the sub-optimal conditions in the greenhouse. The temperature in the greenhouse was highly variable and it is assumed that these adverse environmental conditions resulted in a high degree of stress for the donor plants and affected grain filling. Consequently, the yield was low, restricting the number of replications possible from each treatment group to two

or three. Furthermore, the embryos which were cultured from harvested spikes had already been exposed to considerable stress due to the physiological status of the donor plants. Tissue culture systems are stressful environments in any case, so this would be compounded by the previous stresses from the greenhouse. It is possible that this has contributed to the low quantities of embryogenic calli generated by the three elite Canadian cultivars in this study. Precocious germination was observed frequently in all cultivars and this may also have contributed to the low levels of embryogenic callus. Table 5 indicates that explants of "Golden Promise", the model cultivar, did generate much higher levels of embryogenic calli than the elite cultivars (more than double that of one other cultivar). The environmental factors cast some doubt as to the quality of the data recorded from this study. The somewhat inadequate number of replications due to lack of harvested material, in addition to the questionable quality of explants derived from that material, also contribute to this doubt.

Only one concentration of copper sulphate was examined in the initiation media. It would have been valuable to try a range of concentrations. It is possible that the concentration used here (10μM) was too low to be of significance to these cultivars. Dahleen (1995) indicates that callus initiation is not dependent on the concentration of copper sulphate in the initiation medium. The increased copper concentrations serve to increase the number of plants regenerated from that callus. In this preliminary study, it was deemed that there were too many parameters outside those controlled within the experiment which would have affected the data to make it worthwhile analysing the

regeneration data. For instance, the prior stresses from the greenhouse conditions and thereafter, overheating in the culture room. The temperature in the culture room was over 30°C and the resulting condensation on the plates was considerable causing a large proportion of the regenerating calli to be unusable. Therefore, this study was not useful in determining whether increased copper sulphate in the initiation medium increases the quantity of green plants regenerated from callus. Furthermore, it was not possible to evaluate the effect of increased copper in the regeneration medium for these cultivars in accordance with the studies by Dahleen (1995).

It was decided not to grow further donor material in the greenhouse due to the factors outlined above. Therefore, subsequent studies attempted to evaluate different donor plant growth locations. Dahleen (1999) examined the environmental effects on "Golden Promise" and "Morex" cultivars in greenhouse and growth cabinet locations. She found that cultivar, location and planting date all had significant effects on the degree of green plant regeneration. She found that plant regeneration rates were higher and less variable from the growth cabinet location. This is probably due to the strict control offered by the growth cabinet environmental conditions. She found that at certain planting dates, the explants from the greenhouse yielded comparable results to those she achieved from the growth cabinet. That being the case, if one was to choose the greenhouse over the growth cabinet, one would be restricted to planting only at specific times and under specific prevailing conditions. Dahleen's study ascertained that the greenhouse is not the optimal location in which to grow donor plants for barley tissue culture (Dahleen, 1999).

However, this study did demonstrate that while differences between the two media types were not significant, the results suggest that differences between cultivars were significant. Other than the data for "Golden Promise", the data generated from this study is not sufficient to permit ranking of the cultivars examined in this study. This would be consistent with the reasoning behind "Golden Promise" being used as a model cultivar in various tissue culture and transformation studies (Bregitzer et al, 1999; Bregitzer, 1992; Lührs and Lörz, 1987; Dahleen, 1999; Wan and Lemaux, 1994; Hagio et al, 1995). If the environmental stress resulted in the low levels of embryogenic calli by the three elite Canadian cultivars, it would appear that "Golden Promise" is perhaps better adapted than the elite cultivars to cope with environmental stress.

4.4 Conclusions

From this study, it can be suggested that genotype or genotype-based tolerance of environmental stress is significant in determining the efficiency of embryogenic callus generation from intact immature barley embryos. "Golden Promise" did not produce significantly higher levels of callus but did yield higher levels of embryogenic callus compared to the other cultivars, possibly because it is a more widely adapted cultivar.

The concentration of copper sulphate in the induction medium did not appear to influence the generation of embryogenic calli and the study did not determine whether it does, in fact, influence the regeneration of plants from these calli.

It is possible that the choice of explant (intact immature embryo) affected the level of embryogenic callus produced. Precocious germination often results from the culture of intact embryos and it was observed in high frequency during this study.

For the next study it was decided to only use one type of initiation medium (MS) since there were no significant differences between media types in this study. It was also decided to grow plants in the field plot instead of the greenhouse and to use microsectioned embryos to alleviate the problem of precocious germination and thus, remove that as a factor which may affect generation of embryogenic calli from explants. It was decided to replace "AC Rosser" (six-row) with "AC Metcalfe" (two-row malting) for subsequent studies since the yield of immature embryos was considerably lower for "AC Rosser" than the other cultivars tested in this study.

5.0 Cultu re of Barley Immature Embryos (Microsectioned) From Field-Grown Donor Plants

5.1 Introductio-n

This study used explants derived from donor plants grown in a field plot to evaluate several parameters which are known from previous studies to have a bearing on the efficiency of barley tissue culture. Since the greenhouse environment was stressful, it was decided to try planting donor plants in the field. The following hypotheses were tested:

1. Cultivar has a significant effect on the efficiency of embryogenic callus generation and regeneration of plants in a barley culture system

2. Explant preparation influences generation of embryogenic calli

3. Explant preparation influences regeneration of plants from calli

4. The composition of the regeneration medium influences regeneration of plants

5. The more embryogenic calli produced, the greater the regeneration of plants from those calli

In order to test these hypotheses, donor plants of four cultivars were grown in the field to provide explants for culture initiation. One initiation medium and three regeneration media types were tested. The study was conducted in two phases. Phase one was the culture initiation and callus generation phase and phase two was the regeneration of green plants from the induced callus.

5.2 Materials and Methods

5.2.1 Growth of Donor Plants and Harvest of Spikes

Plants of four Canadian barley cultivars ("Oxbow", "Golden Promise", "AC Rosser" and "AC Bacon") were grown from seed in the Agriculture and Agri-Food Canada field plot (Winnipeg, MB: April, 1998 and April, 1999) in accordance with standard procedure (Watson, pers comm., 1998). Seed was sown once per week over a four week period to reduce the number of mature spikes harvested at any one time, thereby reducing the storage time between harvest and culture. The plot was weeded regularly. No fertiliser was applied and growing conditions were good. Spikes were harvested and handled in the same manner described in section 4.2.1.

5.2.2 Embryo Culture

The embryos were prepared and cultured in the same manner described under section 4.2.2 with the following modifications:

1. Only one initiation medium was used (MS) - see appendix 1 for media composition and preparation
2. Explants were not intact immature embryos. In this study, three different microsections were evaluated. Scutellum only (s-only) explants were immature embryos from which the embryonic axis had been removed with the scalpel. Coleoptilar (c-s) explants were immature embryos where the lower half of the embryo, including the embryonic axis, was removed using the scalpel. Only the scutellar section was cultured.

   Longitudinally bisected (l-b) explants were immature embryos which were halved through the embryonic axis using the scalpel. In all cases, the explants were cultured with the scutellum side down. Figures 4a, 4b and 4c illustrate how the immature embryo explants were prepared.

Figure 4a Preparation of "s-only" Barley Immature Embryo Explant

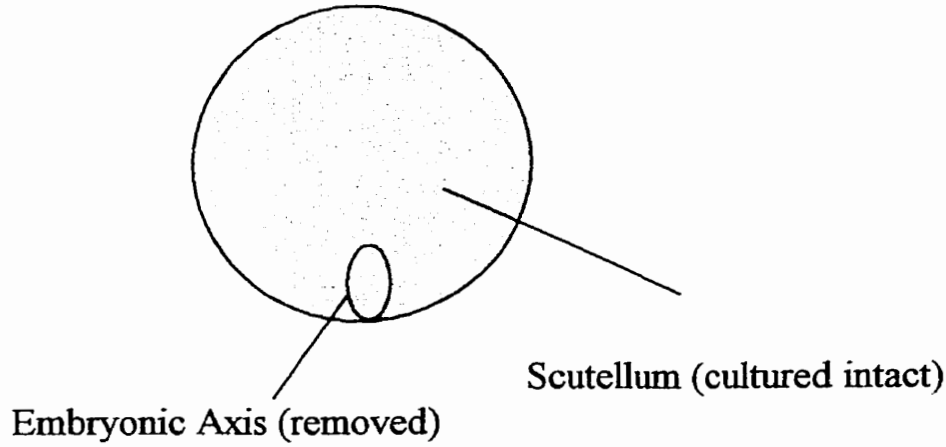


Figure 4b Preparation of "c-s" Barley Immature Embryo Explant

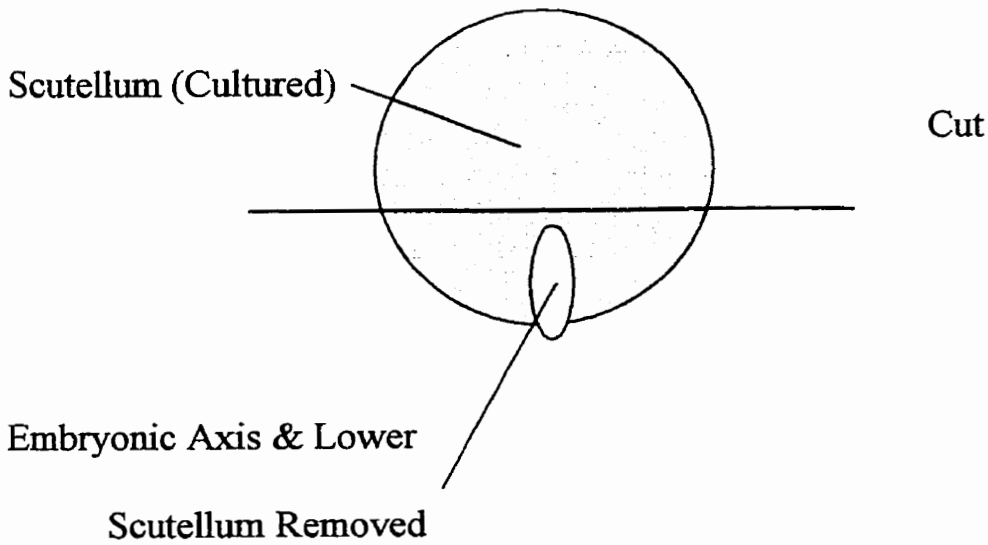
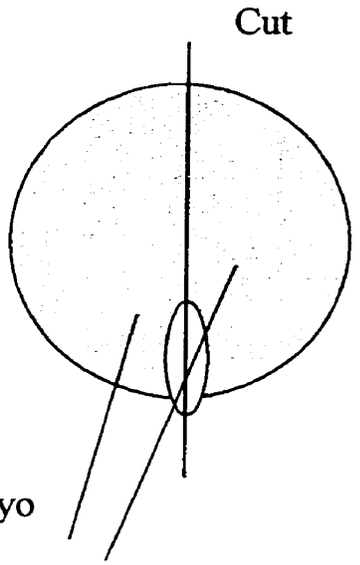


Figure 4c Preparation of "l-b" Barley Immature Embryo Explant



Each Half of the Embryo

(including axis and scutellum)

Cultured Separately

1. Harvested embryos were divided equally with one third of the embryos assigned to each treatment (s-only, c-s and l-b). Approximately 17 embryos were cultured per plate. Approximately 6 replicates for each treatment were obtained and exact numbers were recorded.

2. After 6 weeks under dark conditions at room temperature (27°C), the callus was scored as embryogenic or non-embryogenic as described in section 4.2.3. For each plate of calli, the calli were divided as evenly as possible between each of the

three regeneration media types (regular, FHG and regular plus copper sulphate) and the plates were sealed with Parafilm® then placed in the culture room under light conditions (300μE m^2 s^{-1}) at 20°C for 6 weeks or until sufficient organogenesis permitted transfer to soil. Since each plate sometimes contained both embryogenic and non-embryogenic callus, and we wanted to record whether regenerated plants were from embryogenic or non-embryogenic calli. Calli of the type present in the lowest quantity were circled with permanent marker on the underside of the plate. As before, the calli were sub-cultured every three weeks. After 14 weeks from placing in the culture room, the remaining plates containing non-regenerated calli were discarded.

5. The regenerated plants were potted up in Sunshine Mix® and grown to maturity in the growth cabinet. When pots were labeled, it was recorded on the tag whether the plantlet had been derived from embryogenic or non-embryogenic callus. Growth cabinet lighting was fluorescent (120 μmol m^2 s^{-1}) and incandescent (200 μmol m^2 s^{-1}). The light and dark regime was 8hrs dark and 16hrs light. The temperature was 17°C.

5.2.3 Data Collection and Analysis

For each cultivar and treatment tested for efficiency of embryogenic callus production on the MS initiation medium (phase 1), we recorded the total number of explants cultured for each treatment, the number of replicates of each treatment, the percentage of explants successfully producing callus and the percentage of these calli which were embryogenic, by replicate.

For phase 2, the regeneration study, we recorded the exact number of calli plated on each of the regeneration media for each microsection treatment, the number of plants regenerated from embryogenic calli and the number of plants regenerated from non-embryogenic calli, also from each microsection treatment.

As in section 4.2.4, the data did not fit a normal distribution so an arcsine transformation of the square root of the raw data was performed prior to further analysis. This data did not fit a normal distribution either so t-tests were calculated by hand on the raw data as described in section 4.2.4.

5.3 Results and Discussion

The average percentage of explants successfully producing callus for each treatment and each cultivar tested, and the average percentages of those calli which were embryogenic for each cultivar are recorded in Tables 6 - 9. Paired t- tests were conducted on the raw data within each cultivar to determine whether there were significant differences between the three microsection treatments in formation of total calli and / or embryogenic calli. For the regeneration phase of this study, t-tests were calculated to ascertain whether there were significant differences between microsection, regeneration medium and / or cultivar in the regeneration of green plants from embryogenic calli.

Table 6 Number of Microsectioned Barley Immature Embryo Explants Cultured and Numbers of Calli Generated for "AC Bacon"

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Generating Calli ± Std. Err.	Average % of Total Calli which was Embryogenic ± Std. Err.
s-only	78	6	85.5 ± 7	76.8 ± 7
c-s	126	7	91.2 ± 7	51 ± 8
l-b	109	6	97.3 ± 2	43.7 ± 14

Table 7 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Numbers of Calli Generated for "Golden Promise"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Generating Calli ± Std. Err.	Average % of Total Calli which was Embryogenic ± Std. Err.
s-only	38	2	63 ± 37	50 ± 50
c-s	38	2	54.5 ± 45	100 ± 0
l-b	39	2	57.2 ± 43	50 ± 50

Table 8 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Numbers of Calli Generated for "AC Metcalfe"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Generating Calli ± Std. Err.	Average % of Total Calli which was Embryogenic ± Std. Err.
s-only	87	6	97.9 ± 2	97.2 ± 3
c-s	77	5	90.8 ± 8	93.1 ± 4
l-b	74	4	94 ± 4	95.6 ± 4

Table 9 Number of Microsectioned Barley Immature Embryo Explants Cultured and Numbers of Calli Generated for "Oxbow"

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Generating Calli ± Std. Err.	Average % of Total Calli which was Embryogenic ± Std. Err.
s-only	86	6	100 ± 0	86 ± 8
c-s	99	6	74 ± 14	90 ± 4
l-b	104	6	90.9 ± 8	85 ± 7

In this study there was a deviation from the usual immature embryo size used. For all cultivars excluding "AC Metcalfe" immature embryos between 1.0 and 2.0 mm were used. However embryos or sections of embryos of this size from the "AC Metcalfe" cultivar did not generate callus. Slightly larger embryos (between 2.5 and 3.5 mm) were able to generate callus in a similar fashion to the other three cultivars and it was decided to use these larger sizes when "AC Metcalfe" explants were being prepared. This would indicate that the size of the embryo cannot necessarily be used as an indication of developmental stage in embryonic tissues. Also, in this study, fewer replicates were achieved for "Golden Promise" since considerably less seed was produced by "Golden

Promise" in comparison to the other three cultivars ("AC Bacon", "AC Metcalfe" and "Oxbow") when donor plants were grown in the field.

Tables 10 - 13 contain the results of the t-tests comparing explant effects in the formation of total calli (including embryogenic and non-embryogenic calli) within each cultivar.

Table 10 Paired T-Test Results Between Explant Microsection Types Within "AC Bacon" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0.7	11	-
s-only vs l-b	1.7	10	-
c-s vs l-b	1.07	11	-

Table 11 **Paired T-Test Results Between Explant Microsection Types Within "Oxbow" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Microsections Compared	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
s-only vs c-s	2.05	10	-
s-only vs l-b	1.2	10	-
c-s vs l-b	1.16	11	-

Table 12 **Paired T-Test Results Between Explant Microsection Types Within "Golden Promise" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Microsections Compared	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
s-only vs c-s	0.19	2	-
s-only vs l-b	0.15	2	-
cs vs l-b	0.05	2	-

Table 13 Paired T-Test Results Between Explant Microsection Types Within "AC Metcalfe" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	1	9	-
s-only vs l-b	1.11	8	-
c-s vs l-b	0.4	7	-

Tables 10 - 13 show that no significant differences were observed (at the 5% level) in the formation of total calli from microsectioned explants within any of the cultivars. It would appear that microsectioned explants do not vary in their ability to form calli. Further t-tests were conducted to determine whether microsectioned explants of each cultivar were significantly different in their ability to form embryogenic calli. Tables 14 - 17 contain the results of those t-tests.

Table 14 Paired T-Test Results Between Explant Microsection Types Within "AC Bacon" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	1.6	11	-
s-only vs l-b	2.31	10	*
c-s vs l-b	0.5	11	-

Table 15 Paired T-Test Results Between Explant Microsection Types Within "Oxbow" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0.51	10	-
s-only vs l-b	0.1	10	-
c-s vs l-b	0.7	10	-

Table 16 Paired T-Test Results Between Explant Microsection Types Within "Golden Promise" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	1.41	2	-
s-only vs l-b	0	2	-
c-s vs l-b	1.41	2	-

Table 17 Paired T-Test Results Between Explant Microsection Types Within "AC Metcalfe" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0.9	11	-
s-only vs l-b	0	8	-
c-s vs l-b	0.8	7	-

Table 14 shows that the AC Bacon s-only explant gave significantly higher levels of embryogenic calli than the l-b explant (mean of 77% to a mean of 44% respectively) when tested at the 5% level. However, this was not significant at the 1% level. Since none of the explants from "Oxbow", AC Metcalfe" or "Golden Promise" showed any significant differences, it appears that explant type is not a significant factor in the generation of embryogenic calli from microsectioned explants.

Since explant microsection did not significantly affect formation of callus (total or embryogenic calli), more t-tests were conducted to determine whether cultivar affects the levels of total calli formed or the levels of embryogenic calli. To evaluate the cultivar differences the total data for each cultivar were pooled over explant treatments. Table 18 shows the results of those tests for the total calli produced and Table 19 shows the results of the t-tests for the embryogenic calli produced.

Table 18 Paired T-Test Results Between Cultivars in Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)

Cultivars Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
AC Bacon vs Oxbow	0.5	35	-
AC Bacon vs Golden Promise	1.89	23	-
AC Bacon vs AC Metcalfe	0.8	32	-
AC Metcalfe vs Oxbow	1.02	33	-
AC Metcalfe vs Golden Promise	2.14	19	*
Golden Promise vs Oxbow	1.7	22	-

Table 19 Paired T-Test Results Between Cultivars in Formation of Embryogenic Calli

Cultivars Compared	|t|	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
Oxbow vs AC Bacon	4.19	35	**
AC Bacon vs Golden Promise	0.5	23	-
AC Metcalfe vs AC Bacon	5.9	32	**
AC Metcalfe vs Oxbow	2.03	33	-
AC Metcalfe vs Golden Promise	1.48	19	-
Oxbow vs Golden Promise	4.7	22	**

The cultivars were again significantly different from each other. In the formation of total calli (Table 18), "Metcalfe" gave significantly higher levels of calli at the 5% level of significance, than "Golden Promise". That difference was not significant at the 1% level. However, when cultivars are compared for efficiency in forming embryogenic calli from explants (Table 19), it is clear that "Oxbow" and "AC Metcalfe" formed significantly higher levels of embryogenic calli than "AC Bacon" (at the 1% level). "AC Metcalfe" also produced greater quantities of embryogenic calli than "Golden

Promise" (over 90% for all three explant microsections - Tables 8 and 7 respectively) but the difference was not significant at the 5% level. "Oxbow" was also significantly better than "Golden Promise" (at the 1% level). "Oxbow" and "AC Metcalfe" did not form significantly different levels of embryogenic calli.

Overall, the differences between explant microsection treatments were not statistically significant and that being the case, it can be said that "AC Metcalfe" and "Oxbow" were the cultivars capable of generating the greatest quantities of embryogenic calli, in terms of average percentages of embryogenic calli produced, followed by "Golden Promise", and lastly, "AC Bacon" (Tables 8, 9, 7 and 6 respectively). One would suppose then that "AC Metcalfe" would be the cultivar to regenerate the greatest quantity of plants from that embryogenic calli. However, Table 20 shows that only 20% of the "Metcalfe" embryogenic calli regenerated green plants. 30% of "Oxbow" embryogenic calli regenerated plants, 9% of "Golden Promise" embryogenic calli regenerated plants, and 14% of embryogenic calli from "AC Bacon" regenerated plants. Figure 5 shows plates of regenerating plants from embryogenic calli. Calli from "AC Metcalfe" c-s sections are in the top plate, the bottom left plate contains calli from "Oxbow" c-s sections and the bottom right plate contained "Golden Promise" l-b microsection calli. It should be noted that only one albino plant was produced from this study. This plant was not included in the analyses of regeneration data. All plants included in the data analysis were green.

Table 20 Percentage of Embryogenic Calli Regenerating Green Plants*

Cultivar	**% Embryogenic Calli Regenerating Green Plants (over all explants and regeneration media)**
Oxbow	30
AC Metcalfe	20
AC Bacon	14
Golden Promise	9

* Using the Sum of Plants Regenerated From Each Explant and the Sum of Plants Regenerated From Each Regeneration Medium for All Four Cultivars

Figure 5 Regeneration from Embryogenic Calli of "AC Metcalfe", "Oxbow" and "Golden Promise" Explants

Since the regeneration data was not normalised by the arcsine transformation, t-tests were calculated by hand to compare differences between the explant treatments, regeneration media and cultivars. Tables 21 - 24 contain the results for the comparison of regeneration media in the regeneration of green plants from calli of "Oxbow", "AC Bacon", "AC Metcalfe" and "Golden Promise" s-only, c-s and l-b explants. Since there

were no significant differences between regeneration media types at the 1% level, the data was then pooled over regeneration media to test explants and these results are contained within Table 25. Data was further pooled over explant treatments to test for significant differences between cultivars and these results are provided in Table 26. Figure 6 shows regenerated "AC Metcalfe" plants in comparison to one grown from breeders' seed (control).

Figure 6 **Regenerated "AC Metcalfe" (Left to Right: Control Plant, Plant from "s-only" Explant, Plant from "l-b" Explant and, Plant from "c-s" Explant)**

Table 21 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "Oxbow"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	1.34	10	-
R vs R+Cu	s-only	1.07	10	-
FHG vs R+Cu	s-only	0.05	10	-
R vs FHG	c-s	0.06	10	-
R vs R+Cu	c-s	0.002	10	-
FHG vs R+Cu	c-s	0.05	10	-
R vs FHG	l-b	2.97	10	*
R vs R+Cu	l-b	1.6	10	-
FHG vs R+Cu	l-b	0.77	10	-

Table 22 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "AC Bacon"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	0.91	12	-
R vs R+Cu	s-only	1.29	12	-
FHG vs R+Cu	s-only	0.93	12	-
R vs FHG	c-s	0.56	12	-
R vs R+Cu	c-s	0.004	10	-
FHG vs R+Cu	c-s	0.56	12	-
R vs FHG	l-b	0	12	-
R vs R+Cu	l-b	1.18	12	-
FHG vs R+Cu	l-b	1.18	12	-

Table 23 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "Golden Promise"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	1.41	2	-
R vs R+Cu	s-only	1.41	2	-
FHG vs R+Cu	s-only	0	2	-
FHG vs R	c-s	5.12	2	*
R vs R+Cu	c-s	1.42	2	-
FHG vs R+Cu	c-s	1.98	2	-
R vs FHG	l-b	0	2	-
R vs R+Cu	l-b	0	2	-
FHG vs R+Cu	l-b	0	2	-

Table 24 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "AC Metcalfe"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	1.54	10	-
R vs R+Cu	s-only	1.81	10	-
FHG vs R+Cu	s-only	1.39	10	-
R vs FHG	c-s	1.4	8	-
R vs R+Cu	c-s	0.82	8	-
FHG vs R+Cu	c-s	0.73	8	-
R vs FHG	l-b	2.69	6	*
R vs R+Cu	l-b	2.8	6	*
FHG vs R+Cu	l-b	1.2	6	-

Table 21 shows that regeneration from the R regeneration medium was significantly higher in the l-b explant of "Oxbow" than from the FHG medium (at the 5% level only). There were no significant differences between regeneration media at the 5% level for "AC Bacon" (Table 22). Table 24 ("AC Metcalfe") shows that the l-b section regenerated better on R medium than on FHG or R+Cu media at the 5% level. The following table (Table 25) contains the t-test results for the percentage of plants regenerated from embryogenic calli tested between explant microsections for each cultivar, using pooled regeneration media data.

Table 25 **Paired T-Tests Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Explant Microsections for "Oxbow", "AC Bacon", "AC Metcalfe" and "Golden Promise" (Using Pooled Regeneration Media Data)**

Cultivar	**Explant Treatment Compared**	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
Oxbow	s-only vs c-s	0.02	34	-
Oxbow	s-only vs l-b	1.2	34	-
Oxbow	c-s vs l-b	1.1	34	-
AC Bacon	s-only vs c-s	0.02	40	-
AC Bacon	s-only vs l-b	1.8	40	-
AC Bacon	c-s vs l-b	1.9	40	-
AC Metcalfe	s-only vs c-s	1.02	31	-
AC Metcalfe	s-only vs l-b	1.06	28	-
AC Metcalfe	c-s vs l-b	0.2	25	-
Golden Promise	s-only vs c-s	2.23	16	*
Golden Promise	s-only vs l-b	1.6	16	-
Golden Promise	c-s vs l-b	2.6	16	*

Table 25 shows that for "Oxbow", "AC Bacon" and "AC Metcalfe cultivars there were no significant differences between explant treatments on the regeneration of green plants. However, the table also shows that for "Golden Promise", significantly higher regeneration (at the 5% level) was observed from the c-s microsection explant in comparison to the l-b and s-only sections. Since no explant microsections were significant at the 1% level, the data was pooled over explants to conduct t-tests for cultivar effects on regeneration. The results of these tests are contained within Table 26.

Table 26 Paired T-Tests Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Cultivars (Using Pooled Explant and Regeneration Media Data)

Cultivars Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
Oxbow vs AC Bacon	2.5	112	*
Oxbow vs Golden Promise	3.84	79	**
Oxbow vs AC Metcalfe	1.54	97	-
AC Bacon vs Golden Promise	1.6	85	-
AC Bacon vs AC Metcalfe	0.98	103	-
Golden Promise vs AC Metcalfe	2.43	70	*

Table 26 shows that cultivar was significant in determining the efficiency of regeneration. "Oxbow" was significantly more efficient than "AC Bacon" (at the 5% level) and "Golden Promise" (at the 1% level). "AC Metcalfe" was significantly more efficient

than "Golden Promise" at the 5% level, and "Oxbow" and "AC Metcalfe" were not significantly different to each other in their ability to regenerate plants from calli.

Since it is known that embryogenic calli are responsible for the regeneration of plants, it would seem probable that the cultivar and treatment that produced the most embryogenic calli would in turn, regenerate the most plants. When 193 (97%) plants were regenerated from embryogenic calli and only 6 were regenerated from non-embryogenic calli, it is clear that embryogenic callus is the best type of callus to regenerate plants. However, even though embryogenic calli are produced, it is obvious from this study that not all of these calli go on to regenerate plants. When the cultivar and treatment that generates the greatest quantity of embryogenic callus does not regenerate a similar proportion of plants, it becomes clear that there must be another factor (or factors) responsible for inducing regeneration from somatic embryos on calli. Bregitzer (1992) found that callus cultures which appeared to be embryogenic frequently were not when working with 15 different genotypes. It would seem that a larger proportion of embryogenic calli from "Oxbow" retained the capability of regenerating roots and shoots to form plants while a much smaller proportion retained that capability in "AC Metcalfe". This could be due to a number of reasons.

What was observed in this study was the result of certain cultivars (specifically, "Oxbow" and "Metcalfe") having greater totipotency than the others ("Golden Promise" and "AC

Bacon") under the observed conditions. Even so, "Oxbow" and "AC Metcalfe" did not exhibit similar plant regeneration levels.

It is not inconceivable that the stress of the culture process reduces the totipotency of the cultivars evaluated here to a varying degree. Another reason why this might be the case is due to the action of certain plant growth regulators (PGRs) and the competency of cells. Plant growth regulators such as auxins and cytokinins are responsible for moderating the growth of plants and in culture systems, auxins (such as 2,4-Dichlorophenoxyacetic acid) are added to the media to promote callus or root formation and cytokinins, such as Kinetin are added to promote shoot formation. In cereals, for embryogenesis to occur an induction period is required in the presence of exogenous auxin while the development and maturation of the plants occur in the absence of exogenous auxin. The PGRs act as genetic switches which initiate or inhibit the transcription of certain genes. If there is too high a concentration of auxin in the induction medium, it may inhibit the transcription of genes necessary to the developmental process. Genotypes may vary in their ability to store exogenously applied auxin so when the culture is transferred to a hormone-free medium for maturation and regeneration, the culture still behaves in a manner characteristic of the presence of auxin. If this is the case for certain genotypes, perhaps a different type of auxin which may be stored less efficiently, would generate better results. In addition, it may be possible to mediate the effects of auxin carryover by using a different PGR to stimulate regeneration (for example gibberellins which can conduct the same role as auxin in some

cases or cytokinin in the regeneration medium). Another reason for the high frequency of embryogenic callus and low frequency of regenerated plants from the callus, could be the actual nutritional content of the media and / or preparation of the media (Bregitzer, 1998). In this study casein hydrolysate was used in the media as a nitrogen source. Lührs and Lörz (1987) found that casein hydrolysate increased the frequency of embryogenic callus formation and that there were benefits to increasing the thiamine and inositol concentrations. While the frequency of embryogenic callus formation was increased, it was not determined whether that callus was regenerable or not. Different results could be obtained using different concentrations of these components, or a different nitrogen source. With regard to barley, it is also likely that different results would be obtained with different cultivars. It might be possible in the future to tailor media composition to cultivar to optimise cultivar-specific protocols.

Since the explants most commonly used in monocot tissue culture contain both meristematic and differentiated cells (for instance, immature zygotic embryos), the stage of development and the physiological condition of the explant are of critical importance. It is interesting to note that there were initially problems when introducing "AC Metcalfe" immature embryos into culture due to inconsistencies with size and ability to form unorganised callus in comparison to the other cultivars. Furthermore, the high levels of embryogenic callus the cultivar eventually produced did not generate equivalent levels of regenerated plants. Perhaps further studies are required on "AC Metcalfe" alone to determine the optimal immature embryo size for culture and regeneration.

5.4 Conclusions

From this study, it was determined that cultivar had a significant effect on the efficiency of embryogenic callus production in a barley culture system. The study also determined that cultivar is significant in the regeneration of green plants from embryogenic calli.

Explant preparation did not influence the generation of total calli or embryogenic calli alone. However, within the "AC Bacon" cultivar, the s-only explant was significantly better than the l-b explant in the production of embryogenic calli at the 5% level.

Explant preparation was not significant alone, in the regeneration of green plants from embryogenic calli. However, within the "Golden Promise" cultivar, embryogenic calli from the c-s microsectioned explant had regenerated significantly greater quantities of green plants at the 5% level of significance, than the s-only and l-b sections.

The composition of the regeneration media did not significantly influence the regeneration of green plants from embryogenic calli in general. However, within the "Oxbow" cultivar and l-b microsectioned explant, the R medium was significantly better than FHG (at the 5% level) in the regeneration of green plants from embryogenic calli. In the "Golden Promise" cultivar, c-s explant, the FHG medium was significantly better than the R medium at the 5% level. In "AC Metcalfe" for the l-b explant, the R medium

was significantly better than both the FHG and R+Cu media at the 5% level. From this study, it did not appear that the R+Cu regeneration medium (Dahleen, 1995) improved regeneration of green plants from embryogenic calli.

The more embryogenic calli produced, the greater the quantity of green plants regenerated from the embryogenic calli. However, equivalent quantities of plants to embryogenic calli are not regenerated. This suggests that some other factor(s) is / are responsible for the regenerative capabilities of the calli.

To summarise, it seems that when donor plants are grown under field conditions, efficiency of barley tissue culture is highly cultivar-specific. The study suggests that it is not one factor, or a few isolated factors that determine the efficiency of the culture, rather it is the interaction of those factors. To conduct a barley tissue culture study, one may have to decide on a cultivar and based on the interactions described here, then decide on explant and regeneration medium to tailor the system specifically to that cultivar.

For the next study it was decided to use the same initiation medium (MS). It was also decided to grow donor plants in the growth cabinet instead of the field while keeping all other factors and conditions the same as those for this study. This would allow us to compare the efficiency of callus production and plant regeneration from microsectioned

explants between two different growth conditions for the donor plants (field and growth cabinet). The same three microsectioned explants were used.

6.0 Barley Immature Embryo Culture Using Growth Cabinet-Grown Donor Plants

6.1 Introduction

This study used explants derived from donor plants grown in a growth cabinet to evaluate several parameters which are known from previous studies to have a bearing on the efficiency of barley tissue culture. The studies we carried out prior to this, evaluated the effects of donor plant growth conditions, and various explant and media choices. This will extend the knowledge gained from first two studies by allowing us to evaluate the efficiency of callus production and plant regeneration when donor plants are grown under considerably more defined conditions than those in the first two studies. A particular benefit of this study, where donor plants are grown in the growth cabinet, is that the researcher can set the specific conditions for growth of the donor plants and this is something that can easily be repeated allowing further studies or repeats of this study, to be conducted under the exact same conditions (something which can be difficult to achieve when donor plants are grown in a field plot or greenhouse). For this study, the following hypotheses were tested:

1. Cultivar has a significant effect on the efficiency of embryogenic callus generation and regeneration of plants in a barley tissue culture system

2. Explant preparation influences generation of embryogenic calli alone and through interaction by cultivar

3. Explant preparation influences regeneration of plants from calli alone and through interaction by regeneration medium

4. Composition of the regeneration medium influences regeneration of plants alone and by interaction of cultivar and / or explant

5. The more embryogenic calli produced, the greater the regeneration of plants from that callus

In order to test these hypotheses, donor plants of four cultivars were grown in the growth cabinet to provide explants for culture initiation. One initiation medium and three regeneration media types were tested. The study was conducted in two phases. Phase one was the culture initiation and callus generation phase and phase two was the regeneration of green plants from the induced callus.

6.2 Materials and Methods

6.2.1 Growth of Donor Plants and Harvest of Spikes

Plants of four Canadian barley cultivars ("Oxbow", "Golden Promise", "AC Metcalfe" and "AC Bacon") were grown from seed in the growth cabinet in accordance with standard procedure (Jordan, 1998). The growth cabinet had fluorescent light at 200 μE. The light and dark regime was 8 hours dark and 16 hours light. The temperature was 17°C.

Breeder's seed was sown about 3 cm deep in 18 cm diameter plastic pots with extra drainage in Sunshine Mix® potting medium (Bizimungu, 1998). Four seeds were sown per pot and upon seeding, and once per week thereafter, 5g of Plant Prod® water soluble fertiliser (20% nitrogen, 20% phosphorous and 20% potassium) was added to the soil surface of each pot and then watered in (the exact volume of water applied to each pot was not measured). Plants were watered every 3 days. Approximately 14 days after anthesis, spikes were examined daily for caryopses at the required stage and harvested as described in section 4.2.1.

6.2.2 Embryo Culture

The embryos were prepared and cultured in the same manner described under section 4.2.2 with the same modifications outlined in section 5.2.2.

6.2.3 Data Collection and Analysis

This was conducted in the same manner described in section 5.2.3. Any contaminated plates were discarded and not included in the analyses. In particular, four plates of "Golden Promise" (two c-s and two l-b) were contaminated and therefore, the replicates for this cultivar and its treatments were much fewer than those of the other cultivars. One plate of "AC Bacon" (c-s explant) and one of "Oxbow" (s-only explant) were also contaminated.

6.3 Results and Discussion

The average percentage of explants successfully producing callus for each treatment and each cultivar tested, and the average percentages of those calli which were embryogenic are recorded in Tables 27 - 30. The t-statistic was calculated and tested at the 5% level of significance within each cultivar to determine whether there were significant differences between the three microsection treatments within each cultivar in production of total calli (Tables 31 - 34).

Table 27 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Number of Calli Produced for "AC Bacon"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Producing Callus	Average % of Total Calli which was Embryogenic
s-only	46	4	98 ± 2	37 ± 8
c-s	31	2	100 ± 0	76.7 ± 23
l-b	44	3	92 ± 8	42 ± 16

Table 28 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Number of Calli Produced for "Golden Promise"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Producing Callus	Average % of Total Calli which was Embryogenic
s-only	41	4	100 ± 0	98.5 ± 1
c-s	28	2	47.5 ± 45	100 ± 0
l-b	36	2	89 ± 1	45 ± 34

Table 29 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Number of Calli Produced for "AC Metcalfe"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Producing Callus	Average % of Total Calli which was Embryogenic
s-only	309	15	93 ± 3	100 ± 0
c-s	288	14	80 ± 6	100 ± 0
l-b	301	14	86 ± 6	100 ± 0

Table 30 **Number of Microsectioned Barley Immature Embryo Explants Cultured and Number of Calli Produced for "Oxbow"**

Explant Cultured	Total Number of Explants Cultured	Number of Replicates (plates)	Average % of Explants Producing Callus	Average % of Total Calli which was Embryogenic
s-only	46	4	82 ± 12	63 ± 22
c-s	64	5	66 ± 18	79 ± 13
l-b	68	4	86 ± 10	37.5 ± 15

Tables 31 - 34 contain the results of t-tests conducted on the raw data comparing the explant microsection types of each cultivar for significant differences in the formation of calli (embryogenic and non-embryogenic calli).

Table 31 **Paired T-Test Results Between Explant Microsection Types within "AC Bacon" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	1.16	4	-
s-only vs l-b	0.89	5	-
c-s vs l-b	0.99	3	-

Table 32 **Paired T-Test Results Between Explant Microsection Types within "Golden Promise" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	1.63	4	-
s-only vs l-b	12.78	4	**
c-s vs l-b	1.28	2	-

Table 33 **Paired T-Test Results Between Explant Microsection Types within "AC Metcalfe" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)**

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	2.00	27	-
s-only vs l-b	1.21	27	-
c-s vs l-b	0.67	26	-

Table 34 Paired T-Test Results Between Explant Microsection Types within "Oxbow" Cultivar in the Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli)

Microsections Compared	t-statistic	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
s-only vs c-s	0.86	7	-
s-only vs l-b	0.28	6	-
c-s vs l-b	1.11	7	-

Tables 31 - 34 show that there were no significant differences between explant microsection types in the formation of total calli (embryogenic and non-embryogenic calli) for the cultivars evaluated here, with the exception of "Golden Promise" (Table 32, page 86) which shows a significant difference at the 1% level between the s-only and l-b explants. This seems surprising because the average percentages of total calli produced were 100% and 89% respectively. However, there was no significant difference between s-only and c-s explant (47.5%). This is explained by the extremely low variance for both the s-only and l-b sections (variances of 0 and 1.56 respectively). Replicates of both explants produced either 100% calli or very close to 100% and therefore, the very low variance of the replicates (four replicates for s-only and only two replicates for l-b) makes the difference appear highly significant. Tables 35 - 38 show the results of t-tests

comparing those explant microsections for significance in the formation of embryogenic calli for each cultivar.

Table 35 Paired T-Test Results Between Explant Microsection Types Within "AC Bacon" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	|t|	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	2.19	4	-
s-only vs l-b	0.35	5	-
c-s vs l-b	1.63	3	-

Table 36 Paired T-Test Results Between Explant Microsection Types Within "Golden Promise" Cultivar in the Formation of Embryogenic Calli

Microsections Compared	|t|	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0.82	4	-
s-only vs l-b	2.22	4	-
c-s vs l-b	2.29	2	-

Table 37 **Paired T-Test Results Between Explant Microsection Types Within "AC Metcalfe" Cultivar in the Formation of Embryogenic Calli**

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0	27	-
s-only vs l-b	0	27	-
c-s vs l-b	0	27	-

Table 38 **Paired T-Test Results Between Explant Microsection Types Within "Oxbow" Cultivar in the Formation of Embryogenic Calli**

Microsections Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
s-only vs c-s	0.77	7	-
s-only vs l-b	1.09	6	-
c-s vs l-b	2.41	7	*

Tables 31 - 34 show that there were no highly significant differences between explants in the formation of total calli (embryogenic and non-embryogenic) for any cultivar evaluated here. There were no significant differences between explant microsections for the "AC Bacon, "Golden Promise" and "AC Metcalfe"cultivars. The "Oxbow" results (Table 38) show that the c-s microsection generated significantly higher quantities of embryogenic calli than the l-b section at the 5% level only. The c-s section was not significantly different to the s-only explant at the 5% level. This ties in with the results in Table 41. Since there were no highly significant differences between explant microsection types in the formation of embryogenic calli, the explant data was pooled to do t-tests on the cultivars to determine whether there were significant differences between cultivars in the formation of total calli (Table 39) and / or the formation of embryogenic calli (Table 40).

Table 39 **Paired T-Test Results Between Cultivars in Formation of Total Calli (Includes Embryogenic and Non-Embryogenic Calli) -Using Pooled Explant Microsection Data**

Cultivars Compared	|t|	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
Oxbow vs AC Bacon	2.35	20	*
Oxbow vs Golden Promise	0.52	19	-
Oxbow vs AC Metcalfe	1.13	54	-
AC Bacon vs Golden Promise	1.08	15	-
AC Bacon vs AC Metcalfe	2.52	50	*
AC Metcalfe vs Golden Promise	0.21	49	-

Table 40 Paired T-Test Results Between Cultivars in Formation of Embryogenic Calli -Using Pooled Explant Microsection Data

Cultivars Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
Oxbow vs AC Bacon	1.09	20	-
Oxbow vs Golden Promise	1.72	19	-
Oxbow vs AC Metcalfe	4.03	54	**
AC Bacon vs Golden Promise	2.88	15	*
AC Bacon vs AC Metcalfe	6.25	50	**
Golden Promise vs AC Metcalfe	1.42	49	-

Table 39 shows that there were significant differences at the 5% level between "Oxbow" and "AC Bacon" and between "AC Bacon" and "AC Metcalfe" cultivars in the formation of total calli from microsectioned explants. This also ties in with the data presented in Tables 27 - 30. Tables 27 - 30 show that "AC Bacon" explants did overall, produce more calli. However, that calli was not as embryogenic as the calli produced by the other three cultivars evaluated here and this is described in Table 40 which clearly shows that there were significant differences between cultivars in the formation of embryogenic

calli. This is similar to what was observed in section 5.3 where donor plants were grown under field plot conditions. Specifically, this study shows that "AC Metcalfe" explants generated significantly higher levels of embryogenic calli than "Oxbow" and "AC Bacon" at the 1% level. "Golden Promise" was also significantly better than "AC Bacon" although this was at the 5% level of significance only.

Phase two of this study, examined regeneration from the calli produced during phase one. Table 41 shows the percentage of embryogenic calli regenerating green plants using data pooled for all explants and regeneration media. Figure 7 shows regenerated plants from embryogenic calli of "AC Bacon" with one plant grown from breeders' seed (control).

Figure 7 **Plants Regenerated "AC Bacon" (Left to Right: Control Plant, Plant from "s-only" Explant, Plant from "l–b" Explant and, Plant from "c-s" Explant)**

Table 41 Percentage of Embryogenic Calli Regenerating Green Plants*

Cultivar	**% Embryogenic Calli Regenerating Green Plants (over all explants and regeneration media)**
Oxbow	45
AC Metcalfe	8
AC Bacon	8
Golden Promise	49

* Using the Sum of Plants Regenerated From Each Explant and the Sum of Plants Regenerated From Each Regeneration Medium for All Four Cultivars

Table 41 shows that a much higher percentage of embryogenic calli from "Golden Promise" and "Oxbow" explants, were able to regenerate green plants than embryogenic calli from either "AC Metcalfe" or "AC Bacon" cultivars. Since the percentages of calli regenerating plants from "Golden Promise" and "Oxbow" are quite similar, and the percentages of calli regenerating plants from "AC Bacon and "AC Metcalfe" are exactly the same, it would appear that embryogenic calli from those cultivars are very similar in

their regenerative capacity. It is very interesting to note that for "AC Metcalfe", the cultivar which produced 100% embryogenic calli, of those calli, only 8% regenerated plants. T-tests were conducted on the raw data to determine whether there were significant effects on plant regeneration between each of the regeneration media types. Table 42 contains the results of those t-tests comparing regeneration media effects on plant regeneration from embryogenic calli for each explant microsection within the cultivar "Oxbow". Table 43 contains the results for "AC Bacon", Table 44 contains the results for "Golden Promise" and Table 45 contains the results for the cultivar "AC Metcalfe".

Table 42 **Paired T-Test Results for Percentage of Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "Oxbow"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	0.95	6	-
R vs R+Cu	s-only	0.89	6	-
FHG vs R+Cu	s-only	0.39	6	-
R vs FHG	c-s	1.78	8	-
R vs R+Cu	c-s	1.10	8	-
FHG vs R+Cu	c-s	1.12	8	-
R vs FHG	l-b	1.98	6	-
R vs R+Cu	l-b	1.30	6	-
FHG vs R+Cu	l-b	1.05	6	-

Table 43 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "AC Bacon"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	1.97	6	-
R vs R+Cu	s-only	0.28	6	-
FHG vs R+Cu	s-only	1.15	6	-
R vs FHG	c-s	0	2	-
R vs R+Cu	c-s	0	2	-
FHG vs R+Cu	c-s	0	2	-
R vs FHG	l-b	1.23	4	-
R vs R+Cu	l-b	1.24	4	-
FHG vs R+Cu	l-b	0	4	-

Table 44 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only", "c-s" and "l-b" Explant Microsections for "Golden Promise"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	0.26	4	-
R vs R+Cu	s-only	1.06	4	-
FHG vs R+Cu	s-only	0.99	4	-
R vs FHG	c-s	0.62	2	-
R vs R+Cu	c-s	1.42	2	-
FHG vs R+Cu	c-s	1.42	2	-
R vs FHG	l-b	0.89	2	-
R vs R+Cu	l-b	1.42	2	-
FHG vs R+Cu	l-b	1.42	2	-

Table 45 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Regeneration Media Types Within the "s-only" Explant Microsection for "AC Metcalfe"**

Regeneration Media Compared	Explant Treatment	|t|	Degrees of Freedom	Significance (p=0.05)* (p=0.01)**
R vs FHG	s-only	0.91	26	-
R vs R+Cu	s-only	0.66	26	-
FHG vs R+Cu	s-only	0.23	26	-
R vs FHG	c-s	2.42	26	*
R vs R+Cu	c-s	1.02	26	-
FHG vs R+Cu	c-s	1.73	26	-
R vs FHG	l-b	2.98	26	**
R vs R+Cu	l-b	1.52	26	-
FHG vs R+Cu	l-b	2.72	26	*

Tables 42 - 44 show that embryogenic calli of "Oxbow", "AC Bacon" and "Golden Promise" explants did not differ significantly in their ability to regenerate green plants from calli on each of the three regeneration media. Table 45 however, shows that calli of the c-s and l-b microsections of "AC Metcalfe" regenerated significantly higher numbers of green plants on the FHG regeneration medium than on the R or R+Cu media at 5% and 1% levels of significance. It was decided to pool the regeneration medium data to conduct t-tests for explant microsection effects on regeneration for each cultivar. Table 46 contains the t-test results for explant comparisons of each cultivar.

Table 46 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Explant Microsections for "Oxbow", "AC Bacon", "AC Metcalfe" and "Golden Promise" (Using Pooled Regeneration Media Data)**

Cultivar	**Explant Treatment Compared**	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (p=0.01)****
Oxbow	s-only vs c-s	1.64	25	-
Oxbow	s-only vs l-b	0.34	22	-
Oxbow	c-s vs l-b	2.94	25	**
AC Bacon	s-only vs c-s	1.72	16	-
AC Bacon	s-only vs l-b	1.17	19	-
AC Bacon	c-s vs l-b	1.07	13	-
AC Metcalfe	s-only vs c-s	1.11	82	-
AC Metcalfe	s-only vs l-b	1.59	82	-
AC Metcalfe	c-s vs l-b	0.23	82	-
Golden Promise	s-only vs c-s	1.47	13	-
Golden Promise	s-only vs l-b	1.43	13	-
Golden Promise	c-s vs l-b	0.01	10	-

Table 46 shows that for the "Oxbow" cultivar, embryogenic calli from the l-b explants regenerated significantly higher numbers of green plants than the c-s explants (at the 1% level of significance). There were no significant differences overall between explant microsection type in the regeneration of green plants from embryogenic calli. This data was then pooled to conduct t-tests between cultivars to determine whether cultivar has a significant effect on the regeneration of green plants. The results of these t-tests are contained within Table 47.

Table 47 **Paired T-Test Results for Percentage Plants Regenerated from Embryogenic Calli Tested Between Cultivars (Using Pooled Explant and Regeneration Media Data)**

Cultivars Compared	**|t|**	**Degrees of Freedom**	**Significance (p=0.05)* (P=0.01)****
Oxbow vs AC Bacon	2.08	64	*
Oxbow vs Golden Promise	0.56	58	-
Oxbow vs AC Metcalfe	1.83	163	-
AC Bacon vs Golden Promise	2.11	46	*
AC Bacon vs AC Metcalfe	0.70	151	-
Golden Promise vs AC Metcalfe	1.92	145	-

Table 47 shows that cultivar is significant in determining the efficiency of green plant regeneration from embryogenic calli at the 5% level of significance. Table 41 showed that embryogenic calli of "Golden Promise" were the most regenerable calli (49% of the calli regenerated plants). This is particularly interesting when we know that "AC Metcalfe" generated the greatest quantities of embryogenic calli (significantly higher than "Oxbow" and "AC Bacon"at the 1% level and significantly higher than "Golden

Promise" at the 5% level). Since "AC Metcalfe" did not regenerate significantly different numbers of plants than "Golden Promise" or "Oxbow", obviously "AC Metcalfe" call were not 100% regenerable. "Oxbow" and "Golden Promise" calli must be considerably more regenerable and this is probably due to the same reasons discussed in section 5.3.

From this study it was clear that almost all plants regenerated were from embryogenic calli (94% from embryogenic with only 6% of the total number of plants regenerated from non-embryogenic calli).

6.4 Conclusions

From this study it can be concluded that cultivar has a significant effect on the efficiency of embryogenic callus formation and regeneration of green plants in a barley tissue culture system. "AC Metcalfe" generated significantly higher quantities of embryogenic calli than "Oxbow", "Golden Promise" and "AC Bacon". However, "Golden Promise" calli were more regenerable than calli of the other cultivars.

Explant preparation did not significantly affect the efficiency of total callus formation or embryogenic callus formation. Only one explant of one cultivar ("Oxbow") showed a

significant difference (although the "Oxbow" c-s section was significantly better than the l-b section, this was only at the 5% level and only within that cultivar).

Explant preparation did not influence regeneration of green plants from calli, although within the "Oxbow" cultivar, the c-s explant was significantly better in terms of regeneration than the l-b explant (at the 1% level).

Composition of the regeneration medium did not appear to significantly influence efficiency of regeneration for "Oxbow", "AC Bacon" and "Golden Promise"cultivars. However, within the "AC Metcalfe" cultivar, the FHG medium was significantly better in regenerating plants for the c-s microsections and for the l-b microsections, FHG was significantly better at the 1% level than the R regeneration medium and significantly better at the 5% level than R+Cu.

It was very clear from this study that the hypothesis of: "the more embryogenic calli produced, the greater the regeneration of plants" was not proven. It is clear that the amount of embryogenic calli produced is not the only factor determining whether or not these calli will go on to regenerate plants. The "AC Metcalfe cultivar generated 100% embryogenic calli and yet did not regenerate significantly higher numbers of plants at the 5% level than any of the other three cultivars. This suggests that it is not just the production of embryogenic calli that determines whether plants will be regenerated, rather, it is the regenerative capacity of that calli.

In summary, cultivar is the most significant factor in determining the efficiency of a barley tissue culture. However, when treatments within cultivars are compared, there are instances of one explant microsection or regeneration medium being significantly better than another. This indicates that cultivar and the specific interactions of that cultivar with media components, determines how successful a culture will be. The results suggest that, as observed in Section 5.3, barley tissue culture protocols may have to be designed for each specific cultivar.

7.0 General Summary & Conclusions

When the results of the three studies are considered, it is clear that cultivar has a significant effect on the efficiency of a barley tissue culture (where efficiency of a culture is defined as the ability of explants to form embryogenic calli which, in turn, regenerate green plants). This supports findings from previous studies by Lührs and Lörz, 1987; Hanzel et al, 1985; Bregitzer, 1992; Bregitzer et al, 1998; Dahleen, 1999, and Goldstein and Kronstrad, 1986. In particular, Hanzel et al, (1985) tested ninety-one barley lines and found that only forty-five were able to form callus (embryogenic or non-embryogenic) and only eight lines were regenerable. Of those eight lines, 70% of the regenerated plants were from only two genotypes. In Section 4.0, explants of the "Golden Promise" cultivar produced more than double the quantity of embryogenic calli than "AC Rosser", "AC Bacon", and "Oxbow". This supports the results of previous studies in which "Golden Promise" was evaluated as a model cultivar (Lührs and Lörz, 1987; Tingay et al, 1997; Bregitzer, 1992, Dahleen, 1999, Bregitzer et al, 1999, Wan and Lemaux, 1994 and Hagio et al, 1995). In Sections 5.0 and 6.0, cultivar had a significant effect on the formation of total calli (embryogenic and non-embryogenic) and the formation of embryogenic calli. The results from Section 5.0 show that "AC Metcalfe" explants produced almost twice the quantity of total calli produced by "Golden Promise" explants. "Oxbow" and "AC Metcalfe" both produced significantly higher levels of

embryogenic calli than "AC Bacon" and "Golden Promise" explants. The results from Section 6.0 show that "Oxbow" and "AC Metcalfe" produced significantly higher quantities of total calli than "AC Bacon". "AC Metcalfe" produced significantly higher quantities of embryogenic calli than "Oxbow"and "AC Bacon". "Golden Promise" produced significantly higher levels of embryogenic calli than "AC Bacon".

Due to the cultivar effect on production of total calli and embryogenic calli, cultivar consequently has an effect on the number of plants regenerated from those calli. It is clear, from Sections 5.0 and 6.0, that the majority of plants produced were regenerated from embryogenic calli (almost 100% from both studies), and therefore, the cultivars capable of producing the highest levels of embryogenic calli, would be regarded as the cultivar most likely to regenerate green plants from a barley tissue culture. From the results in Section 6.0, "AC Metcalfe" explants produced 100% embryogenic calli, which was a significantly higher percentage than those produced by "Oxbow". However, only 8% of the "AC Metcalfe" embryogenic calli regenerated plants while 45% of the "Oxbow" embryogenic calli regenerated plants. Therefore, while cultivar does have a significant effect on the efficiency of a barley tissue culture system, there are other factors such as genotype by environment interactions and plant growth regulator effects in addition to media interactions, which determine the regenerative capacity of the embryogenic calli.

There were no significant effects on culture efficiency as a result of explant preparation (intact or microsectioned immature embryos). One point which should be noted is that the optimal immature embryo size for initiation of a barley tissue culture is not constant for all cultivars. These studies illustrate that the "AC Metcalfe" explant had to be almost twice the length of the embryos used for "Oxbow", "AC Rosser", "AC Bacon" and "Golden Promise". Obviously, this shows that between cultivars, physiological genotypic differences dictate the optimal length of immature embryo for successful initiation of a barley tissue culture.

There were no significant effects of initiation media (Section 4.0) or regeneration media (Sections 5.0 and 6.0). While Dahleen (1995) found that increased copper sulphate in the regeneration media increased the number of plants regenerated (for "Hector" and "Excel" cultivars), the cultivars evaluated in this study did not benefit from the 10 μM copper sulphate supplement. This supports studies by Dale and Deambrogio (1979), Lührs and Lörz, 1987 and Hanzel et al (1985) who found that certain initiation and regeneration media were more efficient for some cultivars than others.

Only one albino plant was generated from studies in Section 5.0 and 6.0. This is unusual as the production of albino plants is a considerable problem in barley tissue culture systems (Bregitzer, 1992; Dahleen, 1999; Bregitzer et al, 1999; Wan and Lemaux, 1994). The albino plant was not included in the data analyses since this was the only albino plant. Since our calli were maintained in regenerative conditions for only 14 weeks, this

might explain why there are so few albinos. Albinism can be attributed to somaclonal variation where the plantlets are unable to attain full photoautotrophy, and, while a common occurrence, the effects of this can be minimised by reducing the time spent in culture.

With regard to the effect of growth conditions on the donor material, Section 4.0 shows that highly variable conditions, such as those present in the greenhouse throughout the duration of all three studies, are not conducive to successful tissue culture. Lührs and Lörz (1987) found that environmental conditions strongly influenced the production of embryogenic calli and plant regeneration. Dahleen (1999) found that plant regeneration levels were higher and less variable from the growth cabinet location. Where normal physiological processes in donor plants are disrupted due to sub-optimal conditions, one cannot expect to use explants from those donors to establish a successful culture. Plants were grown in the field plot for the study in Section 5.0 and while conditions were good, the "Golden Promise" cultivar did not appear to be as healthy as the other three cultivars. The yield was lower for this cultivar and this resulted in fewer replicates being established. "Golden Promise" is not a commercially important cultivar in Canada, and its poor performance in the field plot indicates why this might be the case. The defined conditions afforded by the growth cabinet allow studies to be carried under strictly controlled environments permitting accurate replication of results. While the field plot conditions were good in 1998 (Section 6.0), when an attempt was made to replicate this work in 1999, it was not possible to establish a sterile culture without changing the

sterilisation methodology and therefore, a true repeat of the 1998 study could not be conducted. From these studies, it is clear that donor plants should be grown under highly specific conditions to permit standardisation of protocols.

These studies show that clearly, cultivar is the most important factor in barley tissue culture. Since cultivars respond differently and regenerative capacity of embryogenic calli varies between cultivars, barley tissue culture protocols should be tailored to the specific cultivar to be used.

Unfortunately, these studies do not unequivocally answer our questions about which culture protocol is optimal for barley tissue culture. In order to answer these questions, further studies are required which should establish considerably higher numbers of replicates for each treatment. The greenhouse study (Section 4.0) should be repeated under normal greenhouse conditions, which unfortunately were not available while these studies were conducted. However, it is clear from these studies that further work should be directed toward the factor(s) responsible for determining regenerative capabilities of embryogenic calli. If it were possible to increase the regenerative ability of calli, barley tissue culture efficiencies could be significantly improved.

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Appendix 1 - Media Composition

Barley MS and MS+Cu Media

The barley MS media contains the following: MS salts (Murashige and Skoog, 1962), 30 g/l maltose, 1 g/l casein hydrolysate, 1 mg/l thiamine, 2.5 mg/l dicamba, 0.69 g/l proline (filter sterilised), 3.5 g/l phytagar. MS salts are prepared in bulk, in advance and stored in a domestic freezer until use. The proline is filter sterilised using Drummond Self-Locking, 0.8 μm filter. Once the autoclaved litre of media has cooled to a temperature which is warm to the touch (around 40°C), the proline is added under sterile conditions. The media is then briefly agitated to mix the proline thoroughly with the media. The only difference between the barley MS and the barley MS+Cu media is that the MS+Cu media contains 10 μM copper sulphate in addition to the above components. The copper sulphate is added prior to autoclaving.

Regeneration Media (Regular, R+Cu and FHG)

The regular Regeneration medium contains the same components listed above (for barley MS) minus the dicamba and the R+Cu medium contains the same components as the Regular regeneration medium, but is supplemented with 10 μM copper sulphate. The FHG medium contains the same components as barley MS with the following variations: 10-fold less ammonium nitrate in the MS salts, 730 mg/l of glutamine in addition to 0.1

mg/l of BAP (6-benzyl-amino purine). Also, in the FHG medium 62 g/l of maltose are added as opposed to the 30 g/l added to barley MS medium.