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Physiological and Biochemical Aspects of Agrobacterium-Wheat (Triticum Aestivum L.) Interactions

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PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF *AGROBACTERIUM*-WHEAT (*TRITICUM AESTIVUM* L.) INTERACTIONS

by

David L. Parrott, Jr.

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(*Triticum aestivum* L.) Interactions

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Agrobacterium tumefaciens and *A. rhizogenes* are the causal agents of gall or hairy root disease, but normally the bacteria do not cause disease in wheat. However, both bacteria grew without inhibition when exposed to intact or wounded wheat roots or embryos, and they colonized wheat root surfaces to levels similar to dicotyledonous plants. *A. tumefaciens* and *A. rhizogenes* induced 23% cell death after a 1-h exposure to wheat embryo cells grown in 7.4 mM O_2, while the extent of cell death at 2.1 mM O_2 was 8%. Contact with *A. tumefaciens* or *A. rhizogenes* caused cultured wheat embryo and root cells to rapidly produce H_2O_2, which decreased when embryos and roots were cultured at 2.1 mM O_2. Browning and autofluorescence, and an increase in ferulic acid in cell walls, were observed in wheat embryo and root epidermal cells exposed to *Agrobacterium*, but neither lignin nor callose was detected. *Agrobacterium* appeared to induce resistance-like responses in wheat that may limit transformation efficiency.

The inability to regenerate wheat plants using tissue culture has been a limitation to high efficiency transformation. Regeneration via somatic embryogenesis was improved

significantly by simulating the *in ovulo* environment to which the immature wheat embryos are exposed. *Triticum* embryo culture medium (TEC) improved callus formation, somatic embryo formation, and regeneration from somatic embryos while reducing precocious germination when compared to growth on Murashige and Skoog medium. Regeneration frequencies were improved when embryos were cultured at the O_2 concentration found in the wheat ovule (2.1 mM O_2) rather than atmospheric O_2 concentration (7.4 mM O_2).

Agrobacterium-mediated transformation of wheat was limited by tissue necrosis following co-cultivation, and by poor plant regeneration. Reduction of necrosis and increased plant regeneration were accomplished by amending the culture medium with antioxidant compounds and by reducing the O_2 tension in which the wheat embryos were cultured. Twelve days past anthesis (DPA), wheat embryos were co-cultivated with *Agrobacterium tumefaciens* strains WAg11 or EHA101, incubated on TEC medium containing antioxidant compounds (catalase, cysteine and ascorbic acid), and cultured at 2.1 mM O_2 concentrations. Transformation was documented in 6.0% of regenerated *A. tumefaciens* WAg11 exposed wheat plants using the firefly luciferase (*luc*) reporter system.

(112 pages)

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David L. Parrott, Jr.

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CHAPTER 1

INTRODUCTION

Genetic engineering in cereal crop plants, such as rice, corn, and wheat, has importance in providing yield improvement and enhanced resistance to insects and microbes, and tolerance to salinity and drought. *Agrobacterium tumefaciens* and *A. rhizogenes* are useful as vectors for transferring foreign DNA into dicotyledonous plants (Zambryski, 1992). Transformation by *Agrobacterium* has several advantages over other methods, including low copy DNA insertions, stable inheritance of inserts at high frequency, and simplicity (Hansen and Chilton, 1999). However, monocot species, especially cereals, are outside the normal host range for transformation with *A. tumefaciens* (Smith and Hood, 1995). The mechanisms that underlie the intolerance of cereals to *Agrobacterium*-mediated transformation are not known. Such information may help improve the transformation frequency.

Features of *Agrobacterium* and how it operates to transform plant tissues

Agrobacterium tumefaciens and *A. rhizogenes* are Gram-negative, non-sporing, motile, rod-shaped bacteria, closely related to *Rhizobium*, which forms nitrogen-fixing nodules on clover and other leguminous plants (Gelvin, 2003). They are the causal agents of crown gall and hairy root disease, respectively. Most of the genes involved in crown gall (or hairy root) disease are not located on the chromosome of *Agrobacterium* but on a large plasmid, the Ti (tumor-inducing) or Ri (root-inducing) plasmid for *A. tumefaciens* and *A. rhizogenes* respectfully (Figure 1-1a). In the same way, most of the genes that

enable *Rhizobium* strains to produce nitrogen-fixing nodules are contained on a large plasmid termed the *Sym* (symbiotic) plasmid. Thus, the characteristic biology of these bacteria is a function mainly of their plasmids, not of the bacterial chromosome (Zambryski, 1992).

The central role of plasmids in these bacteria can be shown easily by curing of strains. If the bacterium is grown near its maximum temperature (about 30 °C in the case of *Agrobacterium* or *Rhizobium*) then the plasmid is lost and pathogenicity (of *Agrobacterium*) or nodule-forming ability (of *Rhizobium*) is also lost. However, loss of the plasmid does not affect bacterial growth in culture; the plasmid-free strains are functional as saprophytes. It is possible to cure *Agrobacterium* or *Rhizobium* and then introduce the plasmid of the other organism. Introduction of the Ti plasmid into *Rhizobium* causes this to form galls; introduction of the *Sym* plasmid into *Agrobacterium* causes it to form nodule-like structures, although they are not fully functional (Gelvin, 2003).

The *vir* region on the Ti/Ri plasmids is a collection of genes whose collective function is to regulate the excision of the T-DNA region of the plasmid and promote its transfer and integration into the plant genome. The system is induced by signals produced by plants following wounding. Phenolic compounds from wounded plant tissues, such as acetosyringone, activate the *virA* gene, which is a constitutively expressed trans-membrane protein. The activated *virA* gene acts as a kinase, phosphorylating the VirG protein. Vir D1 + D2 have endonuclease activity, and make single-stranded cuts within the left and right borders of the T-DNA.

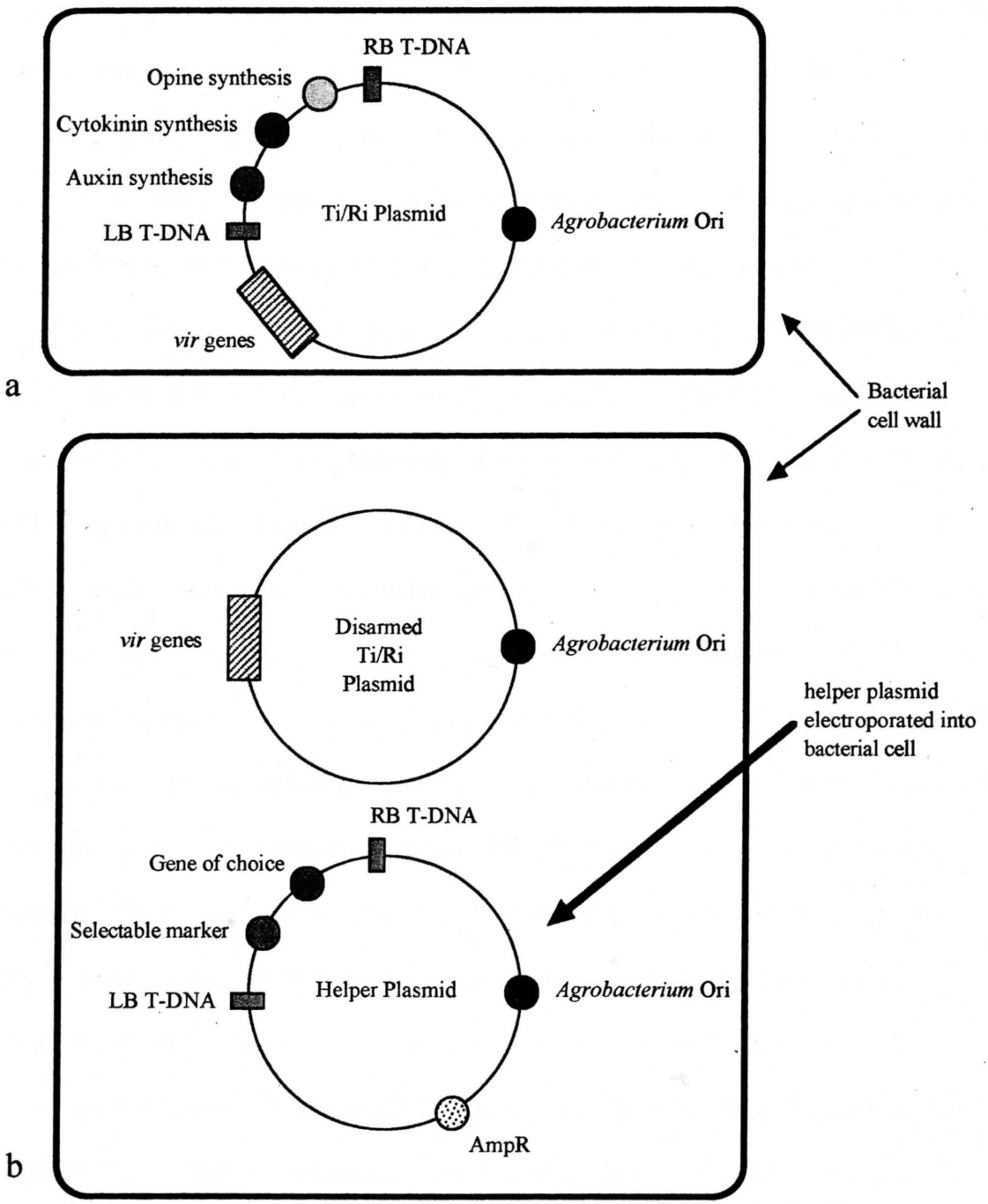

Figure 1-1. Agrobacterium Ti/Ri plasmids. a, Ti/Ri plasmid map b, Binary vector system in *Agrobacterium*. *vir* genes, *Agrobacterium* virulence genes; *Agrobacterium* Ori, plasmid origin of replication; LB, left border of T-DNA; RB, right border of T-DNA. Note that chromosomal DNA is not shown.

VirE acts as a ssDNA-binding protein, protecting the single strand T-DNA region during the transport phase of the process. Once in the plant cell, the complementary strand of the T-DNA is synthesized (Gelvin, 2000), and there is integration into the plant chromosome. These and the other *vir* genes are necessary for transfer of the T-DNA, but they function *in trans*, so none of these genes need to be included in the cloning vectors.

In nature, *Agrobacterium tumefaciens* and *A. rhizogenes* genetically modify the plant by inserting the T-DNA into the host's genomic DNA. The T-DNA carries a number of genes, including one that codes for opine synthesis, two for auxin synthesis, and one for cytokinin synthesis. The hormone biosynthesis genes confer the plant cells with the ability to grow and divide uncontrollably. These genes are often referred to as the oncogenic (*onc*) genes. The opines (amino acid derivatives) octopine or nopaline are used as carbon and nitrogen sources (Zambryski, 1992).

For insertion of foreign genes into plants, modified *Agrobacterium* strains provide all the necessary *vir* functions on modified Ti/Ri plasmids. The *vir* genes reside on a disarmed Ti/Ri plasmid, meaning that the associated T-DNA genes are removed. The T-DNA left and right border regions are placed in a new plasmid, the helper plasmid (Figure 1-1b), and between them any genes to be inserted into the plant (such as the reporter gene luciferase (*luc*) from the firefly, or antibiotic resistance to be conferred to the plant). Typically these two plasmids, or binary vectors are used in *Agrobacterium* mediated transformations because of the helper plasmid's small size and ease of construction. The helper plasmid is electroporated into the *Agrobacterium* strain containing the disarmed Ti plasmid prior to plant transformation (Figure 1-1b).

Wheat-*Agrobacterium* interactions

While dicotyledonous plants are susceptible to *Agrobacterium* transformation, many monocotyledons are not and the processes that differ between the two are not fully documented. However, there are many areas where the transformation process could break down. Listed below are some possibilities:

1) activation of *vir* genes
2) attachment of *Agrobacterium* to plant cells
3) inhibition of growth of the bacterium by chemicals from plant cells
4) induction of resistance mechanisms in plant cells.

As previously mentioned, the *Agrobacterium vir* genes are activated by phenolic compounds produced during the wounding of plant tissues. These compounds act not only as *vir* gene inducers, but as chemotactic agents as well, directing *Agrobacterium* to plant wound sites. It had been thought that suitable phenolic compounds were lacking in monocot plants, preventing transformation from occurring (Messens et al., 1990). However a strong acetosyringone-like *vir* inducer, ethyl ferulate, had been isolated from wheat (Messens et al., 1990). Yet, while the *vir* genes can be activated by ethyl ferulate produced *in vivo*, there was still no successful transformation of wheat cells.

Another possible barrier to successful transformation is attachment of *Agrobacterium* to wheat and maize cells. However, it has been shown by scanning electron microscopy (Graves et al., 1988) that *Agrobacterium* does attach to wheat and maize cells, negating the possibility that adhesion is a limiting step in the transformation process. The adhesion of *Agrobacterium* to the cells of immature wheat embryos *in vitro* is plasmid-independent, meaning that genetic information on the tumor-inducing (Ti)

plasmid is not needed and that chromosomal genes are required (Mooney and Goodwin, 1991).

Growth of *A. tumefaciens* is inhibited by 2,4-dihydroxy-7-methoxy-2H-1, 4-benzoxazin-3(4H)-one (DIMBOA) which is present in maize (Sahi et al., 1990), and also by wheat (Åhman and Johansson, 1994). Therefore, inhibition of *A. tumefaciens* and *A. rhizogenes* growth in the presence of wheat tissues may be one factor involved in the limitation of transformation of wheat tissues.

Reports with grape (Perl et al., 1996), maize (Hansen, 2000) and the woody species aspen and poplar (De Block, 1990) indicate that *Agrobacterium* stimulates cell necrosis with these hosts. A reduction in cell death by the inclusion of antioxidants is correlated with improved transformation efficiency in rice (Enríquez-Obergón et al., 1999), sugarcane (Enríquez-Obergón et al., 1997), sorghum (Zhao et al., 2000), maize (Ishida, et al., 1996), and grape (Hirano et al., 1995).

In other plant-pathogen interactions induced cellular necrosis is involved in the resistance mechanism termed the hypersensitive response (Lamb and Dixon, 1997). An early oxidative burst, in which H_2O_2 is produced, is one of the characteristic events of this response (Dangle, 1998). Baker et al. (1991, 1995) report two phases of H_2O_2 accumulation in plant cells, one occurring immediately with the addition of virulent or avirulent bacteria, and a second phase specific to the avirulent bacteria at 2 to 6 h. The plant cell death that is part of the hypersensitive response is associated with limiting further ingress of the pathogen (Lamb and Dixon, 1997). Increased autofluorescence of plant walls in cells undergoing the hypersensitive response is reported in rice and is attributed to altered phenolic content (Koga, 1994).

Regeneration of wheat via somatic embryogenesis

The *in vitro* culture and regeneration of wheat plants is another potential problem to useful transformation. The transformation process *in vitro* involves small portions of plant tissues, termed explants, rather than whole plants, to be exposed to *Agrobacterium*. While a successful transformation event may take place in a given cell or tissue, without a successful plant culture and regeneration system in place, this transformed cell will not become a whole plant.

Immature wheat embryo tissues were the most receptive tissue for callus induction on MS medium compared with nodal and internodal stem segments, and rachis segments (O'Hara and Street, 1978). Wheat embryos can also be easily induced to undergo somatic embryogenesis, a process by which embryos are produced via somatic, or non-reproductive cells. Because this process creates embryos that mature into plants from single cells, one transformed cell could lead to an entire transformed plant.

The steps to obtaining wheat somatic embryos start with immature whole embryos excised from wheat caryopsis. These embryos, now referred to as explants (small pieces of plant tissue that are cultured), are placed onto a plant culture medium that contains nutrients for plant growth and plant hormones (auxins and cytokinins) to induce the explant cells to produce callus (a mass of differentiated and undifferentiated cells). Not only is callus produced at this stage, but globular-stage embryos are generated as well. The auxins in the culture medium prevent further maturation of the embryos, and it is necessary to subculture the callusing explants to new culture medium that does not contain auxins. It is at this stage that the embryos form heart-shaped, then torpedo-shaped

embryos as are normally seen *in vivo*. These embryos will mature into whole plants, and can be subcultured to soil (Ammirato, 1983).

Regeneration of plant tissues *in vitro* may be accomplished by optimized tissue culture media formulated for specific plant tissues. Initiation and regeneration of plantlets varies depending on initiation medium used (Carman, 1988; Fenell et al., 1996). Murashige and Skoog's (MS) basal medium (1962), developed for the regeneration of tobacco (*Nicotiana tabacum* L.) cells, has been used extensively to regenerate many different species of plants, including wheat. The genotype of the plant and the environment in which it is grown is critical for high callus induction and regeneration from wheat embryos (Hess and Carman, 1998). Modifications to the MS medium have been made (e.g., Maheshwari et al., 1995) to improve induction of embryogenic callus and somatic embryos using immature wheat embryos as explants.

Hess and Carman (1998) experimented with the concept that optimized nutrition for the explant would lead to increased regeneration of transformed plantlets. In transformed tissues, this would increase the number of transformants per explant. They developed a medium that simulated the *in ovulo* fluids to optimize somatic embryogenesis and reduce precocious germination of immature embryos, a process which halts somatic embryogenesis.

Wheat transformation

It has already been established that monocots, including *Allium*, *Asparagus*, *Hordeum*, *Narcissus*, and *Triticum* (Conner and Dommisse, 1992), can be hosts (i.e., the bacteria can colonize roots and may form small tumors) for *Agrobacterium* species.

Wheat has been successfully transformed using *Agrobacterium* as the vector (Cheng, et al., 1997), although transformation efficiencies are quite low. Transformation efficiencies in monocot species via *Agrobacterium* range from 30% in rice (Enríquez-Obregón, 1999) to 1.5% in wheat (Cheng, et al., 1997). Because *Agrobacterium* has proven difficult to use with most monocots, other methods, most notably biolistics, have been developed for gene transfer. Reported transformation efficiencies in cereals using biolistics range from 0.01%-1.0 % (Smith and Hood, 1995).

Research objectives

The overall goal of this research was to determine why *Agrobacterium* interactions with wheat resulted in very low frequency transformation. The broad objectives of this work were to 1) look at wheat-*Agrobacterium* interactions to determine the factors that might be easily altered to reduce defense mechanisms in wheat, thereby increasing transformation efficiencies, and 2) to integrate cutting edge plant cell culturing techniques to successfully regenerate transformed plants. A simple diagram of *Agrobacterium* interactions with wheat is shown in Figure 1-2. More specifically, my null hypotheses are:

1) wheat transformation is not limited by the inability of *Agrobacterium* cells to grow in the presence of wheat cells
2) *Agrobacterium* cannot colonize wheat roots
3) wheat does not respond with defense-like reactions to *Agrobacterium*, including H_2O_2 evolution, plant cell death and cell wall modifications
4) wheat defense responses are not reduced by lowering O_2 tensions in plant tissue culture

5) optimized plant cell culture media and culture environment do not increase the regeneration of wheat plants

6) use of antioxidant compounds, specialized wheat culture medium, and lowered O_2 tensions will not increase *Agrobacterium*-mediated transformation efficiencies in wheat.

Initial experiments were designed to look at bacterial-plant interactions in relation to plant defenses such as bactericidal action. As discussed previously, DIMBOA is present in wheat (Åhman and Johansson, 1994). My first experiments discussed in Chapter 2 were to test whether growth of *A. tumefaciens* and *A. rhizogenes* is inhibited by wheat and whether colonization of the root surface could occur.

There is much evidence of H_2O_2 evolution, plant cell death and hypersensitive responses (HR) upon bacterial contact with dicot plant cells (Lamb and Dixon, 1997; Perl et al., 1996; Hansen, 2000; De Block, 1990). Whether a similar response was occurring in

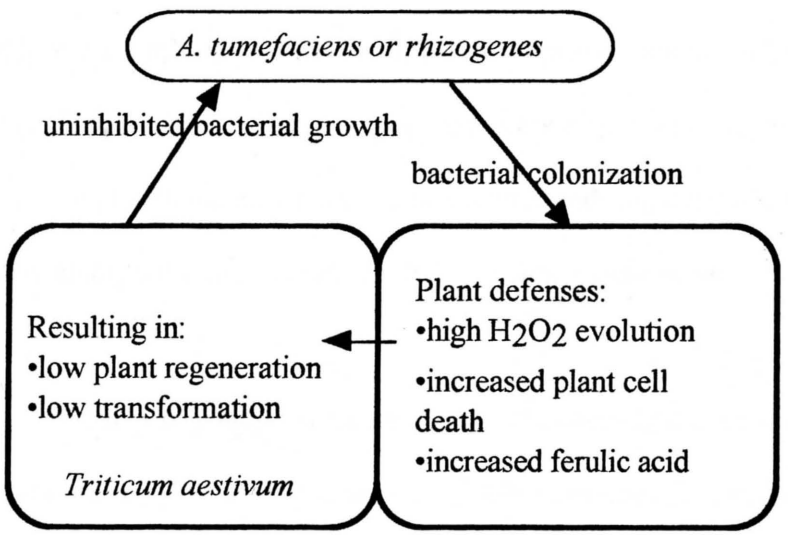

Figure 1-2. A brief summary of interactions between *Agrobacterium* and wheat.

wheat was tested in Chapter 2. The wheat cell walls were examined to determine whether there were physical changes to the structure, such as lignification, callose deposition, or ferulic acid build up. I hypothesized that such modifications may prevent transfer of T-DNA to the plant cell.

Reactive O_2 species accompany the oxidative burst and involve atmospheric O_2 (Lamb and Dixon, 1997). Based on research involving inhibition of cell death caused by tobacco mosaic virus and *Pseudomonas syringae* in tobacco cells grown at low O_2 levels (Mittler et al., 1996), I hypothesized that a reduction in the O_2 tensions would reduce the oxidative burst and HR in wheat caused by *Agrobacterium* exposure, thus reducing plant cell death and necrosis. This hypothesis was tested in chapter 2.

Coincidently, O_2 levels affect plant embryo cell cultures. Normal development of wheat embryos in culture occurs at 2.1 mM O_2 (Carman, 1995). Thus in chapter 3, I performed studies at two O_2 concentrations, 2.1 mM O_2 and 7.4 mM O_2 with the hypothesis that lower 2.1 mM O_2 environment would improve somatic embryogenesis and plant regeneration in wheat. Because plant regeneration may also play an important role in transformation, I tested a new plant tissue culture medium, designed specifically for wheat somatic embryo culture, which would be an improvement for somatic embryo production.

In Chapter 5, I investigated the effects of several antioxidant compounds (Enríquez-Obergón, et al., 1997, 1999; Zhao et al., 2000), lowered O_2 tensions and improved plant regeneration culture media on *Agrobacterium*-mediated transformation of wheat. I hypothesized that a reduction in available O_2 and use of antioxidant compounds,

along with increased regeneration of wheat plants via improved culture medium and culture conditions would increase *Agrobacterium*-mediated transformation of wheat.

Finally, a synopsis of my research and results, and my future study directions are located in the summary, Chapter 5.

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CHAPTER 2

AGROBACTERIUM INDUCES PLANT CELL DEATH IN WHEAT (*TRITICUM AESTIVUM* L.)[1]

The symptoms of gall or hairy root do not occur in the interactions between wheat (*Triticum aestivum* L.), and other monocotyledonous plants, with *Agrobacterium tumefaciens* or *A. rhizogenes*. However, both bacteria colonized wheat root surfaces at similar levels (2.0×10^7 colony forming units/mg root) and grew without inhibition in suspension with intact or wounded wheat embryos or root segments present. Suspension-cultured wheat embryo cells grown in 7.4 mM O_2 displayed 23% cell death after one-h exposure to *Agrobacterium* cells, while the extent of cell death with 2.1 mM O_2 averaged 8%. Cultured wheat embryo and root cells rapidly produced hydrogen peroxide (H_2O_2) when contacted with *A. tumefaciens* or *A. rhizogenes*. Production of H_2O_2 was lower at 2.1 mM O_2 than 7.4 mM O_2. Browning and autofluorescence of epidermal cells of callus derived from wheat embryos and wheat roots was observed after inoculation with *Agrobacterium*. An increase in ferulic acid was detected in the walls of roots exposed to *Agrobacterium*. However, neither lignin nor callose was detected by diagnostic staining methods. These findings suggest that *Agrobacterium* induced a resistance-like response in wheat that may reduce the efficacy of transformation and limit the normal symptom formation.

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INTRODUCTION

Agrobacterium tumefaciens and *A. rhizogenes* produce the symptoms of gall [*11*] and hairy root [*40*], respectively, on dicotyledonous plants and they are useful as vectors for transferring foreign DNA into these susceptible hosts [*43*]. However, galls and hairy roots are not observed upon inoculation of monocotyledons with *Agrobacterium*, and the frequency of transformation by agroinfection is less than with dicotyledons. Genetic engineering in cereal crop plants, such as rice, corn, and wheat, is important for providing enhanced insect, microbial, and herbicide resistance, yield improvement, and salinity and drought tolerance. Transformation by *Agrobacterium* has several advantages over other methods, including low copy DNA insertions, stable inheritance of inserts at high frequency, and simplicity [*20*] and thus ways to improve this process in cereals are desired. Determining the mechanisms that underlie the interactions between *Agrobacteria* and cereals may help improve the transformation frequency.

The processes that differ in the interactions of *Agrobacterium* with monocotyledons and dicotyledons are not fully documented. Scanning electron microscopy reveals that *A. tumefaciens* cells attach to wheat and maize leaves [*17*] negating the possibility that adhesion is a limiting step in the transformation process. The adhesion of *Agrobacterium* to the cells of immature wheat embryos *in vitro* is plasmid-independent, meaning that genetic information on the Ti-plasmid is not needed [*30*] for this stage of the interaction. While attachment has been shown, there are no studies on possible suppression of bacterial growth in the presence of wheat tissues. A strong inhibitor to the growth of *A. tumefaciens*, 2,4-dihydroxy-7-methoxy-2H-1, 4-benzoxazin-

3(4H)-one (DIMBOA), is present in maize [*41*]. DIMBOA also is produced by wheat [*2*]. Consequently, we examined whether *A. tumefaciens* and *A. rhizogenes* would colonize wheat roots and whether compounds present in wheat would inhibit the growth of these bacteria.

Agrobacterium incites plant cell necrosis in several tissues that are difficult to transform: grape [*36*], maize [*19*], and the woody species aspen and poplar [*10*]. A reduction in cell death by the inclusion of antioxidants is correlated with improved transformation efficacy in rice [*13*], sugarcane [*14*], sorghum [*44*], maize [*23*], and grape [*22*]. Since 1997 [*34*], we have been investigating whether cell death also was induced in wheat after challenge with *A. tumefaciens* or *A. rhizogenes.* Wheat embryo cultures were included in these studies because immature embryos are the easiest to manipulate in tissue culture and they have better regeneration potential than other tissues [*7*].

Induced cellular necrosis is typical of the resistance mechanism termed the hypersensitive response and is associated with limiting further ingress of the pathogen [*27*]. An early oxidative burst in which H_2O_2 is produced is one of the characteristic events of this response [*9*]. Baker *et al.* [*3, 4*] report two phases of H_2O_2 accumulation in plant cells, one occurring immediately with the addition of virulent or avirulent bacteria (Phase I), and a second phase specific to the avirulent bacteria at two to six h (Phase II). The walls of cells undergoing the hypersensitive response show increased autofluorescence attributed to altered phenolic content [*24*]. These findings prompted us to examine whether cultured wheat cells produced H_2O_2 upon challenge with *Agrobacterium* and whether the walls of the contacted cells become altered in composition.

In tobacco, a reduction in available O_2 reduces the extent of hypersensitive plant cell death upon challenge with *P. syringae* pv *phaseolicola* [*29*]. Reduced O_2 level is found to improve callus and somatic embryo formation, and to decrease precocious germination of embryos [*7*]. Indeed normal development of wheat embryos in culture occurs at 2.1 mM O_2 [*7*]. Because of the success in the use of antioxidants to reduce cell death and improve transformation, we investigated the effects of lower O_2 on cell death and hydrogen peroxide formation for wheat cells exposed to *Agrobacterium.*

MATERIALS AND METHODS

Bacterial strains

Agrobacterium tumefaciens C58 [*32*], *A. rhizogenes* A4 [*16*], and *Pseudomonas syringae* pv *syringae* B728a [*22*] were grown on Luria broth (LB) medium (10.0 g l^{-1} tryptone, 5.0 g l^{-1} yeast extract, 10.0 g l^{-1} NaCl, pH 7) or low phosphate AB glucose medium [*25, 27*] modified with 20.0 mM MES, 2.0 mM phosphate buffer (pH 7.0), 2.0 g l^{-1} L-glutamate, and lacking yeast extract. Stocks of the bacterial isolates were made by freezing at –80 °C a suspension of an overnight bacterial culture grown for 16 h at 31 °C, in 15% sterilized glycerol.

Bacterial growth and root colonization of Agrobacterium in the presence of wheat tissues

To examine the impact of wheat tissues on bacterial growth, *A. tumefaciens* C 58, *A. rhizogenes* A4, and *P. syringae* B728a were grown in 50 ml low phosphate AB glucose medium [*1*] for 24 h to an OD_{600} of 0.2 ($1x10^8$ colony forming units (cfu) ml^{-1}) at 25 °C in a rotary shaking incubator. Sterile-grown wheat embryos and roots were either

kept intact, were wounded by nicking lightly with a sterile scalpel, or were homogenized (1 g ml^{-1} wheat tissue in TEC medium) on ice using a homogenizer (Polytron, Brinkman Instruments). Bacterial suspensions in AB medium were amended with individual whole, wounded or homogenized wheat embryos or roots using 1 mg tissue ml^{-1}. Non-amended bacterial cultures were used as the controls. At 1 h intervals of growth at 25 °C, 200 µl samples were removed from each of the treatments and the OD was measured at 600 nm and recorded. The measurements were continued until maximum growth was obtained, after approximately 40 h. Blanks for the spectrophotometer were prepared containing low phosphate AB glucose medium and, in the case of the homogenized embryo or root treatment, low phosphate AB glucose medium supplemented with homogenized wheat tissue.

The embryos for these studies were obtained from whole immature embryos removed from kernels 12 d past anthesis (DPA) under sterile conditions. Embryos were cultured for three days in the dark at 25 °C, embryo axis down (to prevent precocious germination), on *Triticum* Embryo Culture (TEC) medium supplemented with 2.0 µM kinetin, 1.0 µM 2,4-D, 2.0 g l^{-1} Phytagel, pH 5.2. TEC medium consists of 2.47 g l^{-1} $MgSO_4$, 0.40 g l^{-1} NH_4NO_3, 0.54 g l^{-1} NH_2PO_4, 0.18 g l^{-1} NaCl, 0.83 mg l^{-1} KI, 6.2 mg l^{-1} H_3BO_3, 22.30 mg l^{-1} $MnSO_4$ $4H_2O$, 8.6 mg l^{-1} $ZnSO_4$ $7H_2O$, 0.25mg l^{-1} $NaMoO_4$ $2H_2O$, 0.025 mg l^{-1} $CuSO_4$ $7H_2O$, 0.025 mg l^{-1} $CoCl_2$ $6H_2O$, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 0.1 mg l^{-1} thiamine HCl, 1.34 g l^{-1} malate, 565.0 mg l^{-1} $(NH_4)_2HC_6H_5O_7$, 100.0 mg l^{-1} casein hydrolysate, 343.3 mg l^{-1} proline, 348.4 mg l^{-1}arginine, 199.7 mg l^{-1} aspartic acid, 438.3 mg l^{-1} glutamic acid, 225.2 mg l^{-1} asparagine, 365.3 mg l^{-1} glutamine, 1.34 g l^{-1} alanine, 2.10 g l^{-1} serine, 262.4 mg l^{-1} leucine, 375.4 mg l^{-1} glycine, 180.2 mg l^{-1}

methionine, 234.2 mg l^{-1} valine, 292.4 mg l^{-1} lysine, 262.4 mg l^{-1} isoleucine, 165.2 mg l^{-1} phenylalanine, 36.7 mg l^{-1} Fe EDTA, and 2.58 g l^{-1} KOH.

To determine whether *Agrobacterium* would colonize wheat roots, surface-sterilized kernels were exposed to overnight cultures (1 x10^8 cfu ml $^{-1}$) of a tetracycline- and kanamycin-resistant *A. tumefaciens* strain WAg11 [*5*] for 5 min and were then planted in a sterile vermiculite and sand mixture (1:1) in enclosed boxes. This strain permitted the use of selective medium to ensure recovery of the *Agrobacterium* used as inoculum. Three boxes, each containing four seeds, were planted. Plants were grown for six d, and then were carefully removed from the soil mixture. Roots were either blotted onto LB medium or washed to remove bacteria. For root blots, roots were pressed firmly onto LB medium with 10 mg l^{-1} tetracycline and 20 mg l^{-1} kanamycin for 10-15 min and removed. Roots to be washed were vortexed for 30 s in 5 ml 20 mM $MgCl_2$. A serial dilution was made in sterile 20 mM $MgCl_2$ and 200 µl of each dilution was plated on LB plates amended with tetracycline and kanamycin. Plates were grown overnight at 31°C, and colonies were counted.

Plant cell death and autofluorescence

Plant cell death was assessed in root and embryo suspension cultures grown in normal and low O_2 tensions. Root suspension cultures were initiated from the roots of 6 d-old, sterile-grown wheat seedlings. The wheat kernels were surface-sterilized using 10% sodium hypochlorite for 20 min, washed with three sterile-water rinses (2 min each) and placed on solid MS medium [*31*] without hormones, but containing 30 g L^{-1}sucrose and 8 g L^{-1} agar, and placed under fluorescent light with an 18 h day-length to raise the

seedlings. Roots were excised under sterile conditions and placed on MS medium supplemented with 30 g L^{-1} sucrose, 1.0 mg L^{-1} 2,4-D, 8 g L^{-1} agar, pH 5.7 to induce callus formation [*33*]. Callus was placed in liquid MS medium with the same additions and shaken at 250 rpm in the dark at 25 °C. Cultures were used at 14 d after transfer in the studies. Root suspensions were also grown in low O_2 concentration, 2.1 mM, in a chamber flooded with nitrogen to displace O_2. O_2 level was maintained using an O_2 sensor (Seatronics, Hatboro, PA) attached to a datalogger (Campbell Scientific, Logan, UT) controlling a solenoid valve to modify the gas mixture. Suspensions were used 14 d after initiation.

To derive the embryo tissue cultures, embryos were allowed to callus on the TEC medium and sections of the callused tissue were placed in suspension culture (25.0 ml liquid TEC medium supplemented with 2.0 µM kinetin, 1.0 µM 2,4-D, pH 5.2, on an orbital shaker at 100 rpm) for 3 d to generate a cell suspension. For cells grown at low O_2, the embryos were grown for 3 d on the TEC medium in the chamber flooded with nitrogen to displace O_2. Cell suspensions were prepared in the low O_2 chamber environment as described above.

Plant cell death was measured using 100 µl of wheat embryo or root suspension culture (0.05 g l^{-1} plant cells in MS or TEC medium) to which either 100 µl of bacterial suspension (OD_{600} 0.2, $1x10^8$ cfu ml^{-1}) or 100 µl cell-free culture filtrate was added. Suspensions of C58, A4 and B728a were used in these studies. At 30 min intervals for 5 h, 10 µl aliquots were removed and placed on a hemocytometer, and 10 µl of 0.01% fluorescein diacetate solution was added. The slide was viewed for the viable fluorescent cells after a 5 min incubation using an Olympus BH-2 microscope with a BH-RFL

fluorescence vertical illuminator, a UG-1 exciter, DM-400 dichroic mirror, and a Y-475 barrier filter. Percentage cell death was calculated based on live versus dead cells counted in five large squares (1/25 square mm) on each half of a hemocytometer.

To examine the effect of bacterial contact on autofluorescence of wheat cell walls, intact wheat embryos, cultured for 3 d on the TEC medium, or intact sterile-grown roots were used. Wheat tissue samples, with or without a bacterial treatment, were placed on a slide with 25 µl double distilled H_2O and were observed using an Olympus BH-2 microscope with a BH-RFL fluorescence vertical illuminator, a BG-1 exciter, DM 500 dichroic mirror, and a O530 barrier filter. Autofluorescence of cell walls was observed under blue fluorescent light (410 nm) as bright yellow [*25*].

To examine lignin in wheat cells, both phloroglucinol and Maule's reagent were used [*38*]. To detect syringyl lignin structures, phloroglucinol was prepared by dissolving 2 g phloroglucinol in 100 ml ethanol and adding 50 ml of 12 M hydrochloric acid. Four drops of this solution was placed on wheat embryo callus cells or whole wheat roots that had or had not been exposed for 2 d to *Agrobacterium*. The same tissues were tested using the Maule test which detects guiacyl lignin [*26*] as a dark red brown color. Wheat tissues were exposed to 60 mM potassium permanganate for 5 min at room temperature, washed with water, then treated with 1.0 M hydrochloric acid. The tissues were again rinsed with water, and 1 ml of 25% ammonium hydroxide was added. As a positive test for lignin deposition, a 5 mg ml^{-1} solution of a mixture of chitin and chitosan in double distilled H_2O was applied to wheat root tissues for 1 h, and samples were taken at 5 min intervals to test for lignin using phloroglucinol and Maule's staining reagents [*38*].

For callose detection, wheat embryo callus, derived as described above, and sterile grown wheat roots were used. The tissues were not inoculated for the controls or were exposed for two d to the bacteria prior to fixing in FAA (90 ml 70% ethanol, 5 ml glacial acetic acid, 5 ml formaldehyde) for 24 h. The fixed cells were rinsed in 70% ethanol for 30 min, transferred to 30% ethanol for 30 min, and finally placed in water for 30 min. The tissues were then transferred to diluted clearing solution (84.6 g sucrose and 10 mg analine blue in 100 ml water) mixed 1:1 with water for 30 min, and finally to 100% clearing solution [*35*]. Tissues were viewed using an Olympus BH-2 microscope with a BH-RFL fluorescence vertical illuminator with a UG-1 exciter, Y-455 dichroic mirror, and a L-435 barrier filter [*35*].

Ferulic acid was detected in wheat roots challenged with *A. tumefaciens* by use of UV fluorescence microscopy. A Zeiss Axioskop 2 fitted with an epifluorescence attachment, technique module FL and filter set 01 (excitation, 365 nm, barrier, 400 nm) was used. The roots of 6 day-old sterile grown plants were exposed to *A. tumefaciens* C 58 for 48 h by the application to the growth matrix of suspension of the stationary bacterial cells cultured on AB medium. Transverse sections, prepared from the roots of the 8-days old seedlings with or without the bacterial inoculum, were suspended in water and viewed with UV light. Under these conditions, the wheat root cell walls fluoresced blue. The sections were treated with a drop of 0.1 M ammonium hydroxide [*21*] and if ferulic acids were present the deep blue fluorescence to changed to an intense dark green.

Assessment of H_2O_2 formation in wheat tissues exposed to bacteria

Whole embryos and root suspension cultures grown at normal and low O_2 concentration were used in the assessment of the production of H_2O_2 upon bacterial challenge. Luminol-dependent chemiluminescence (LDC) [*4*] was used to monitor the evolution of H_2O_2 from the wheat cells.

C58, A4 and B728a cells were grown overnight in AB medium to an OD_{600} 0.2 ($1x10^8$ cfu ml^{-1}). Wheat embryo or root suspension cultures grown at 7.4 mM or 2.1 mM O_2 (500 µl of a 50 mg l^{-1} plant cell suspension in MS or TEC medium) were treated with 100 µl of 16h bacterial culture ($1x10^8$ cfu ml^{-1}) or bacterial culture filtrate in which the bacteria were removed by centrifugation at 10,000 rpm for 3 min, and the medium filtered through a sterile 0.22 µm filter. A stock solution of luminol (32.2 mM in 1.0 M NaOH) was diluted 1:20 in 1.0 M sodium NaOH (pH 7.0) daily, and 100 µl was added to the wheat embryo/bacterial suspensions or the whole embryo/bacterial suspensions just prior to reading. Chemiluminescence was measured using a luminometer (Lumat, Berthold Technologies) over a 10 s period.

Statistical analysis

Bacterial growth, plant cell death, and H_2O_2 production measurements were subjected to repeated measures analysis of variance (SigmaStat 2.0, Jandel Scientific) and considered significant at a p-value < 0.05.

RESULTS

Bacterial growth and root colonization by Agrobacterium

The growth potential of *A. tumefaciens* and *A. rhizogenes* was not impaired by the presence of intact and wounded wheat tissues (for comparison with tobacco, see Appendix A, Fig. A-1). Growth of an incompatible pathogen *P. syringae* B728a was examined for comparison. Each of these bacteria showed increased growth in the media with addition of whole or homogenized plant tissues over growth in low phosphate-AB glucose medium alone (P=0.009 for A4, P=0.006 for C58, P=0.005 for B728a) (Fig. 2-1). The type of tissue provided (whole, wounded, or homogenized embryo or root) did not affect the response (P=0.49 for A4, P=0.1798 for C58, P=0.102 for B728a).

A. tumefaciens WAg11colonized the wheat root surface at approximately 2.6 x 10^7 cfu mg^{-1} of root tissue for six-d-old plants. Blots of the inoculated wheat roots onto growth medium produced bacterial colonies corresponding to imprints from the root surface (data not shown). No colonies were observed from roots of seedlings grown from seeds that were not inoculated with bacteria.

Contact with Agrobacterium elicits plant cell death

To determine whether wheat tissues responded to *Agrobacterium* with cell necrosis, wheat cell viability was assessed using fluorescein diacetate. Exposure of embryo or root-derived callus cells to *A. tumefaciens*, *A. rhizogenes*, and *P. syringae* B728a resulted in increasing levels of plant cell death in a time-dependent manner (Fig. 2-2 a-f). The extent of cell death was less in the root than the embryo cells (10-15% for

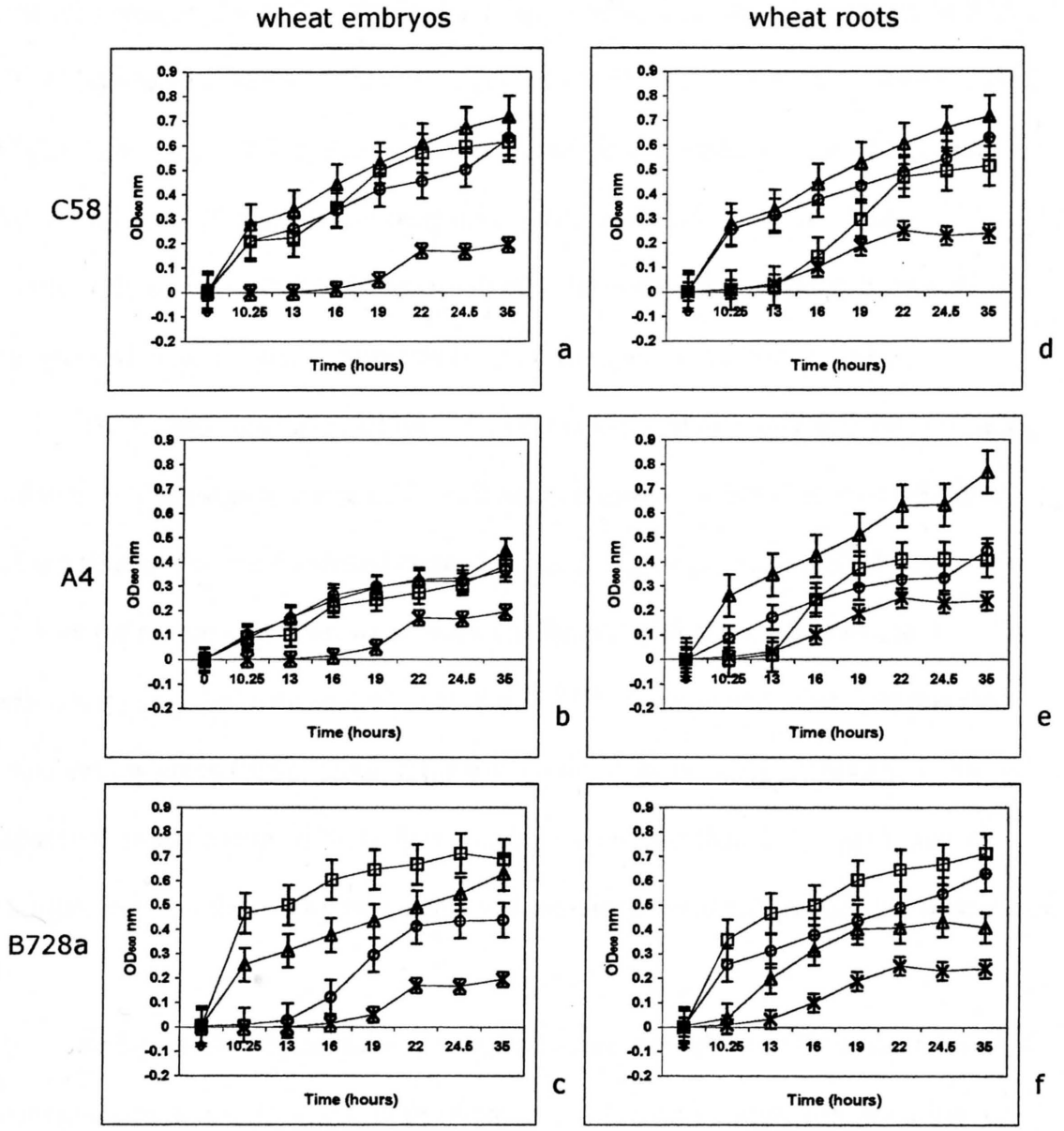

FIG. 2-1. Effect of wheat factors on *Agrobacterium* growth in culture. *Agrobacterium* strains were grown in low phosphate AB glucose medium. Whole, wounded, or homogenized wheat embryo or root tissues were exposed to bacteria. Controls contained no plant material. Wheat embryos were exposed to *A. tumefaciens* C58 (a), *A. rhizogenes* A4 (b), and *P. syringae* B728a (c). Wheat roots were exposed to *A. tumefaciens* C58 (d), *A. rhizogenes* A4 (e), and *P. syringae* B728a (f). Graphs are from one study representative of six separate experiments. Error bars equal standard error from the mean for the six replicates of this one experiment. O bacteria and wounded plant tissues, □ bacteria and whole tissues, Δ bacteria and homogenized plant tissues, X, control, no plant tissues.

root cells compared with 20-25% for the embryo cells). Cell death in response to B728a (10-15%) was less than with the challenges with *A. rhizogenes* A4 and *A. tumefaciens* C 58 (20-25%) (Fig. 2-2, a-c). Culture filtrates also caused similar extents of cell death in the wheat tissues (Fig. 2-2). Lowering the O_2 tension from 7.4 mM to 2.1 mM significantly reduced (P=0.018) the extent of embryo and root cell death both with bacteria and culture filtrate treatments to levels between 6-8% (Fig. 2-2d-f).

The tissues were assessed for other changes related to plant cell death, browning and increased autofluorescence of the cell walls. Exposure to both bacterial cells and culture filtrates for two d induced these changes. A higher proportion of wheat embryo and root cells were visibly browned when challenged with *A. tumefaciens* and *A. rhizogenes* compared with control cells (Fig. 2-3). Irradiation with blue light revealed a bright yellow-green fluorescence of the walls only of the browned embryo and root cells exposed to bacteria (Fig. 2-3b, f). Fewer embryo cells, less than 2 %, were brown or autofluorescent in the control treatments with non-inoculated culture medium (Fig. 2-3c, d).

No lignin-like deposition was observed in roots or wheat embryos after two d of treatment with *A. tumefaciens* C58 or *rhizogenes* A4 or their culture filtrates using phloroglucinol/HCl or Maule's staining techniques. Staining for callose using analine blue also was negative. Treatment of roots and embryos with a 5 mg ml^{-1} solution of a mixture of chitin and chitosan in double distilled H_2O elicited positive staining for lignin with both stains in the epidermal cells after 15 min (data not shown). However, ferulic acid was detected, by noting a change in autofluorescence from blue to green at alkaline pH, in 6 d-old wheat root cells after exposure for two d to *A. tumefaciens* (Fig. 2-4a, b),

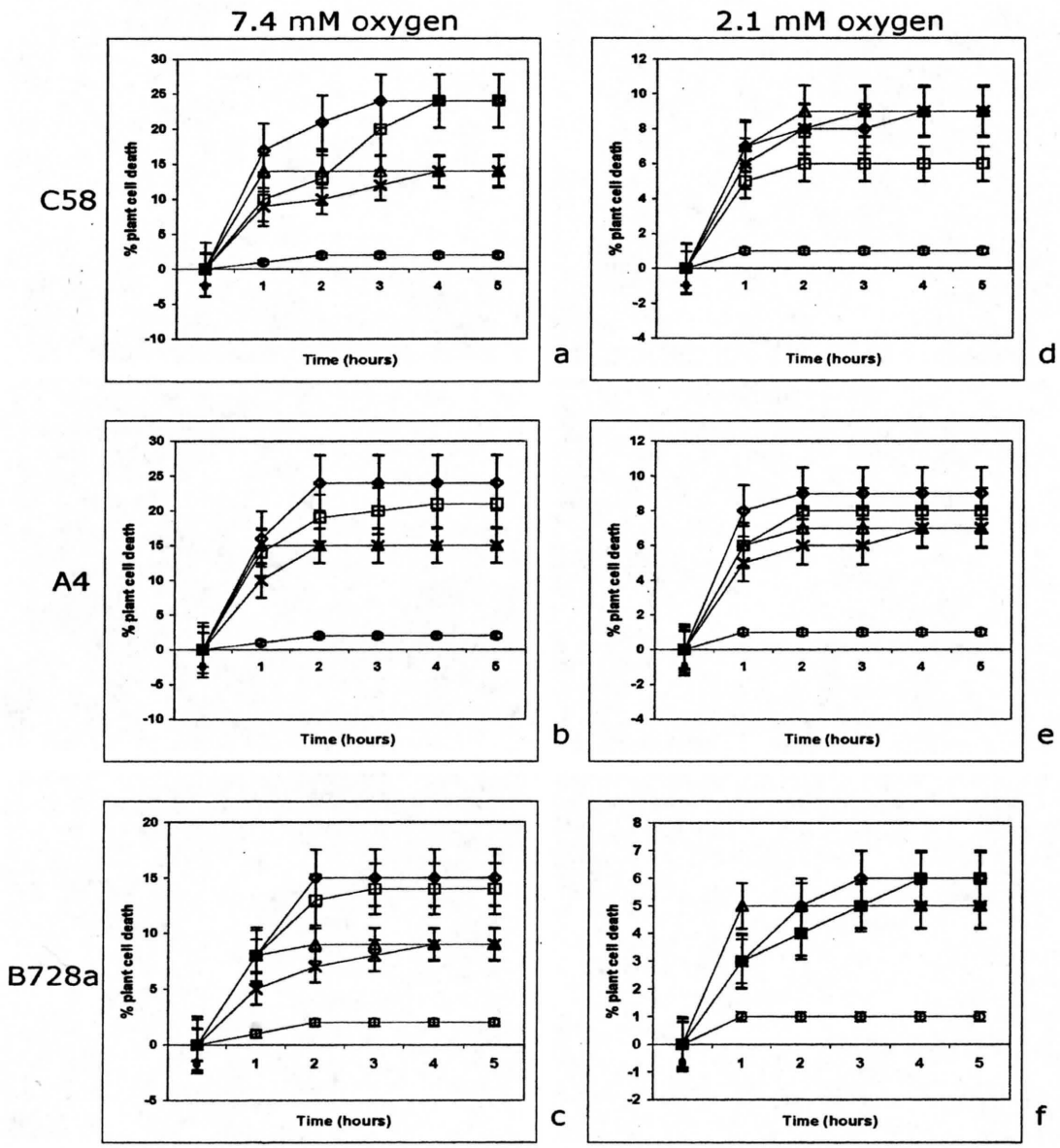

FIG. 2-2. Wheat cell death at 7.4 mM and 2.1 mM O_2 tensions after challenge by *Agrobacterium* or *Pseudomonas* cells. Embryos and roots were exposed to *A. tumefaciens* (a) C58, *A. rhizogenes* A4 (b), and *P. syringae* B728a (c) cells or culture filtrates at 7.4 mM O_2. Wheat embryo and root cell death at 2.1 mM O_2 exposed to *A. tumefaciens* C58 (d), *A. rhizogenes* A4 (e), and *P. syringae* B728a (f) cells or culture filtrates. Control contained neither bacteria nor bacterial culture filtrate. Graphs are from one study representative of 10 separate experiments. Error bars equal standard error from the mean for the 10 replicates of this one experiment. ◊ bacteria and embryo tissues, □ bacterial culture filtrate and embryo tissues, Δ bacteria and root tissues, X bacterial culture filtrate and root tissues, O control.

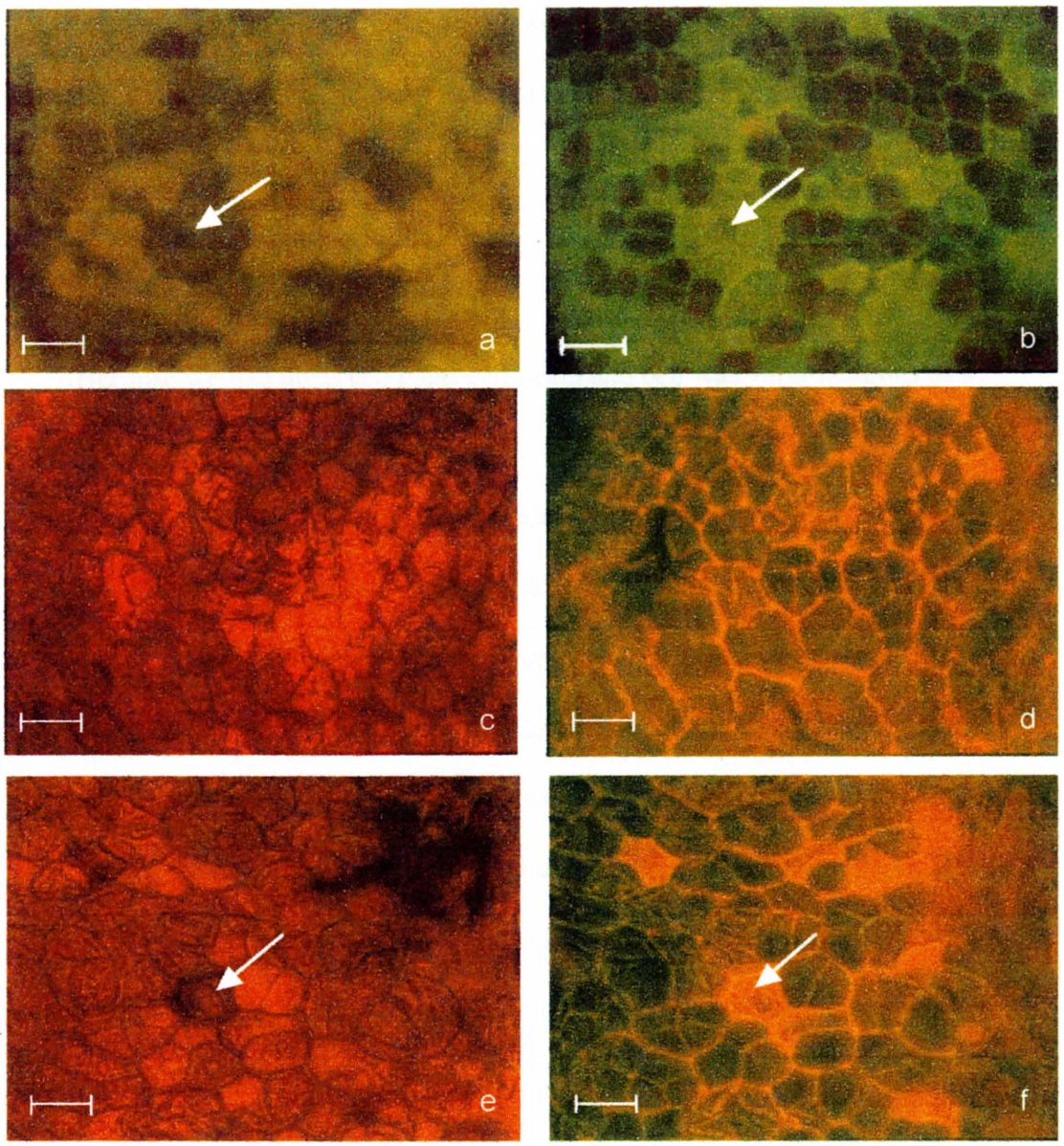

FIG. 2-3. Browning and autofluorescence of wheat embryo and root cells challenged with *A. tumefaciens* C58. (a) light micrograph of embryo cells at 20x. Brown cells are denoted with arrows. (b) Fluorescent micrograph of same cells at 20x. Note that cells at arrow corresponding to the brown-colored cells in the light image have yellow-green fluorescence. (c) Light micrograph of wheat embryo cells treated with non-inoculated culture medium, 20x. (d) fluorescent micrograph of same preparation at 20x. Yellow coloration is from barrier filter O 530. (e) light micrograph of root cells at 20x. Brown cells are denoted with arrows. (f) Fluorescent micrograph of same cells at 20x. Red coloration in cells in (b) and (d) due to fluorescence of chlorophyll. Images representative of six separate experiments, and six fields of view per experiment. Scale bar equal 50 µm.

or *A. rhizogenes* and culture filtrates of *A. tumefaciens* and *A. rhizogenes* (data not shown). Control plants that were not exposed to these bacteria did not show ferulic acid accumulations in the cell walls (Fig. 2-4c, d).

Agrobacterium increases wheat cell H_2O_2 production

Because we had observed plant cell death and cell wall modifications as a response to inoculation with *Agrobacterium*, we determined whether bacterial challenge increased the production of hydrogen peroxide, as has been reported in hypersensitive-responding cells [*27*]. Wheat embryo-derived callus cells produced H_2O_2 rapidly at levels above the control when challenged with *A. tumefaciens, A rhizogenes*, or *P. syringae* (Fig. 2-5a, c). A second burst of H_2O_2 production was observed at between 3.5 and 4 h with the C58 and A4 challenges. Plant cells exposed to bacterial cell-free culture filtrates from both *A. tumefaciens* or *A. rhizogenes* and *P. syringae* also elicited H_2O_2 production (Fig. 2-5b, d). but at significantly lower level ($P=<0.001$ for C58 at both O_2 levels). By lowering the O_2 tension from 7.4 mM to 2.1 mM , there was a significant reduction ($P=0.011$ for C58, $P=0.001$ for A4) in the H_2O_2 produced by plant cells exposed to the *Agrobacterium* cells especially at the 3/5-5 h times (Fig. 2-5c, d). There was no effect of reduced O_2 tension on the production of H_2O_2 after contact of wheat tissues with *P. syringae* B728a.

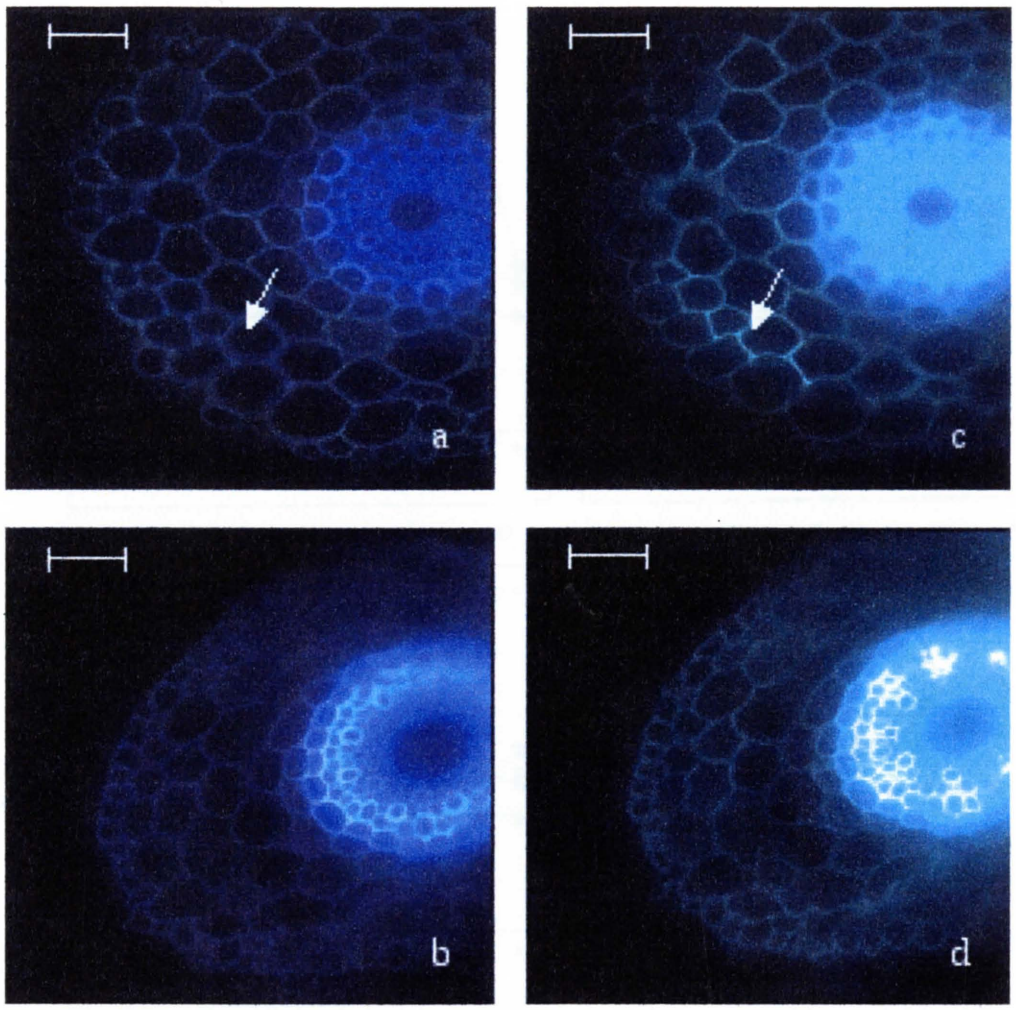

FIG. 2-4. Ferulic acid accumulation in a transverse section from a wheat root exposed for two d to *A. tumefaciens* C 58 at pH 7 (a) and pH 10 (c). A color shift from blue autofluorescence (a) to green (c) upon changing pH from 7 to 10 denotes ferulic acid accumulation as shown by arrows. Control non-inoculated wheat root cells (b, at pH 7.0) and (d, pH 10.0). Images are representative of 12 separate experiments and six fields of view per experiment. Scale bar equal 50 μm.

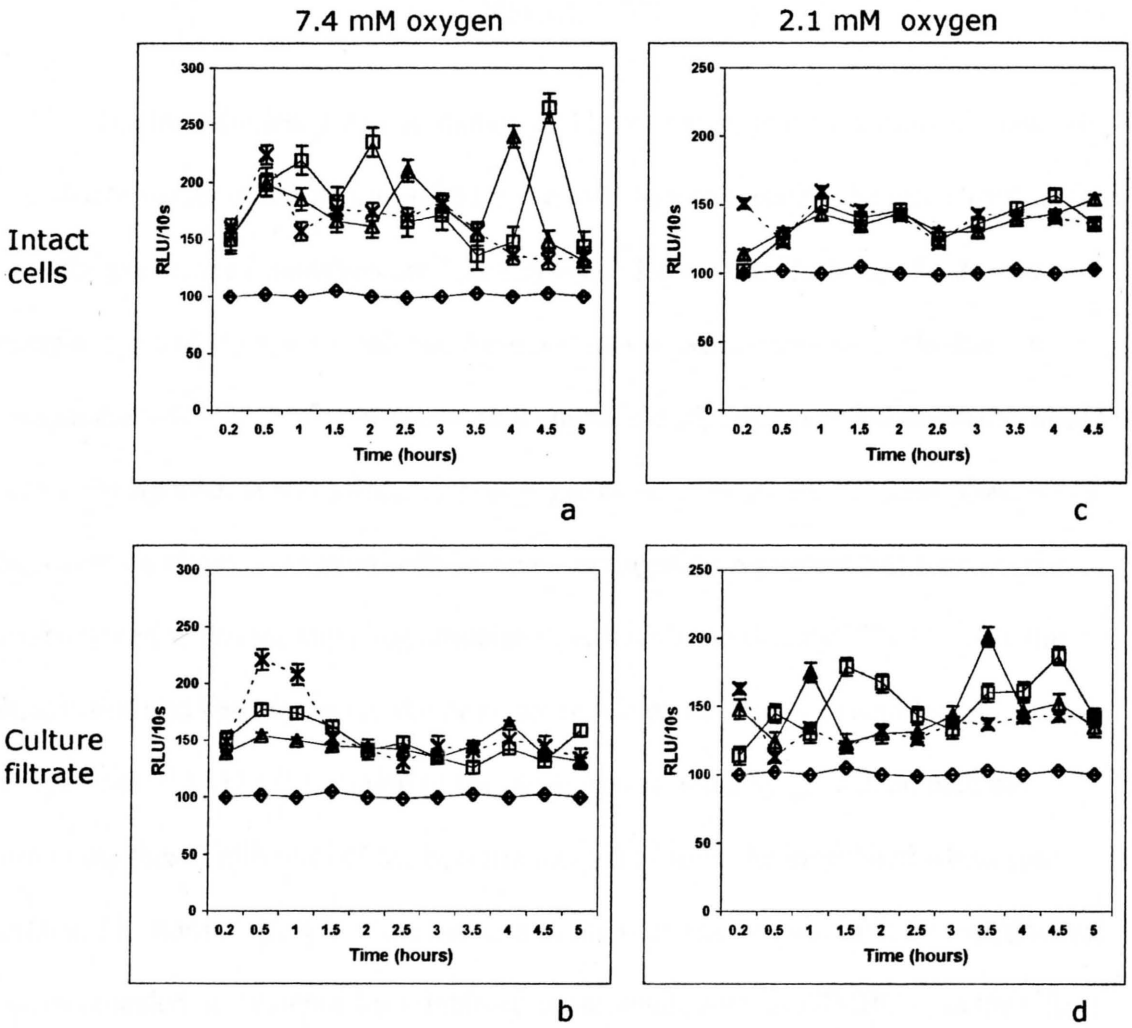

FIG. 2-5. H_2O_2 production after challenge of wheat cells by *A. tumefaciens*, *A. rhizogenes*, and *P. syringae* bacterial cells and bacterial culture filtrates. Luminol-dependent chemiluminescence of wheat embryo cells exposed to intact cells at (a) 7.4 mM O_2 and (c) 2.1 mM O_2, and wheat embryo cells exposed to bacterial culture filtrates at (b) 7.4 mM O_2, and (d) 2.1 mM O_2. RLU= relative light units. Control contained only plant cells, no bacteria. Graphs are from one study representative of 10 separate experiments for each treatment. Error bars equal standard error from the mean for the 10 replicates of this one experiment. □ C58, Δ A4, X B728a, ◊ control.

DISCUSSION

The low efficiency transformation and lack of symptom formation of wheat by *Agrobacterium* does not appear to be related to failure to colonize the wheat root surface or to the presence of inhibitors produced by wounded tissues. Both *Agrobacterium tumefaciens* and *rhizogenes* colonized the surfaces of wheat roots to levels that are comparable with those of saprophytic root colonizers [*6*, *28*]. Such cell densities should permit the *Agrobacterium*-virulence plasmid to be retained by the colonies. Transfer of the virulence plasmid between cells involves conjugation, a process that requires the acylhomoserine lactone signaling associated with high cell density [*37*]. Because the tetracycline and kanamycin resistance genes reside on the binary plasmid in *A. tumefaciens* WAg11 [*5*] retention of the plasmid was noted by growth on medium containing these antibiotics of the bacteria recovered from the inoculated wheat root surface. The normal growth of the bacteria in the presence of intact and wounded wheat tissues revealed no evidence for inhibitory compounds, such as DIMBOA, in the wheat cultivar examined. Rather, in liquid culture with wounded wheat tissues where conditions exist that cause dicotyledonous plants to produce components to activate *vir* gene expression [*43*], the bacteria thrived. However, the wheat cells responded to contact by *Agrobacterium* cells with induction of changes typical of resistance events.

We found that wheat inoculated with *Agrobacterium* increased production of H_2O_2, browned, and displayed altered cell wall composition and higher levels of cellular necrosis. These responses were seen with embryo tissues, which are often used in transformation studies, as well as the plant roots, which would be the site of colonization

of the bacterium in nature. Each of theses events is characteristic of the hypersensitive response observed in incompatible microbial challenges of mono and dicotyledons [*27*]. The level of H_2O_2 production, the extent of cell death and induction of browning were sensitive to O_2 tension, being lower at 2.1 mM, than atmospheric tension, 7.4 mM. Onset of cell death proceeded immediately after challenge of the wheat with either *A. tumefaciens* or *A. rhizogenes*, at a time that was coincident with increased H_2O_2 formation. This timing was distinct from that reported for the hypersensitive response in dicotyledons [*27*] where cell death occurred at a time later than the initial burst of H_2O_2. However, in wheat, we observed that the bean-pathogenic bacterium *P. syringae* pv. *syringae* B728a also displayed early and coincident cell death and hydrogen peroxide formation. We propose that the trigger for cell death may involve a mechanism different from that characterized for the hypersensitive response in race-cultivar interactions [*12*]. In the race-cultivar interactions, the product from a specific avirulence gene in the pathogen is proposed to be detected by a mechanism involving a resistance gene in the host to trigger apoptosis. The avirulence products are thought to be transported into the host cell by a specific transfer system from the bacterial cell [*42*]. However, our finding that wheat cell death was caused by factors in the *Agrobacterium* culture filtrates resembled the effects of extracellular harpins that are produced by some plant pathogenic bacteria and lack the race-cultivar specificity properties [*8*]. Further studies are needed to identify the eliciting factors from *Agrobacterium* because their modification may lead to improved transformation potential.

We examined the nature of the changes in the wheat plant cell walls induced by *Agrobacterium* because such changes may be important in limiting transformation. No

induced lignification or callose formation was observed. This situation differed from resistance in wheat to fungal pathogens [*18*] where lignification of the walls occurred and could account for limited ingress of fungal pathogens into the host cell [*39*]. Callose production was not apparent in the *Agrobacterium*-inoculated wheat root, or embryo cells. However, the increased autofluorescence observed for the necrotic wheat cell walls could be related to increased ferulic acid deposition that was demonstrated in challenged epidermal cells. Whether such modification limits the transfer of genetic material is unknown.

There is now evidence from several systems that links the induction of plant cell death by *Agrobacterium* strains with suboptimal transformation efficiency. In maize [*19*] *A. tumefaciens* was proposed to trigger an apoptosis-like mechanism because by expression of either of two cell death regulator genes, *p35* and *iap* reduced the level of induced cell death. The tissues expressing *p35* - and *iap*- were more conducive to transformation [*19*]. Our observation of browning in the *Agrobacterium*-wheat interaction was similar to that observed in grape [*36*]. Reduction in necrogenesis by inclusion of antioxidants permitted the grape to be transformed by *A. tumefaciens* [*36*]. From preliminary data (Parrot and Carman unpublished data), it seems that the lower O_2 tension, which reduced wheat cell death, also is associated with higher transformation rates by *A. tumefaciens*. This technique could be especially valuable because low O_2 tension is required for maximum growth of embryonic tissues [*7*] and thus this condition would be compatible with regeneration of transformed tissues.

Clearly, the induction of necrosis by *Agrobacterium* in the wheat tissues would prevent those cells from becoming transformed. However, we observed that not all cells

in a treated tissue become necrotic. One possible explanation for the failure of these non-necrotic cells to become transformed is that the generation of the necrotic cells activated systemic resistance in adjacent and even more distant cells. That necrotic cells act as the trigger for systemic acquired resistance effective against bacterial pathogens has been observed for other systems [*15*].

In summary, we find that wheat, like some of the other monocots studied, displayed a necrotic response to contact with *Agrobacterium* isolates. Our findings extend the studies performed by other groups with *A. tumefaciens* of induced plant cell death by *A. rhizogenes*. We propose that modification of the potential *Agrobacterium* elicitors that signal plant cell death, or reduction of O_2 tension during the plant-bacterial interaction, may be studied in more detail, in addition to the published use of antioxidants, to improve *Agrobacterium* transformation of cereals.

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CHAPTER 3

AN IMPROVED MEDIUM FOR WHEAT (*TRITICUM AESTIVUM* L) EMBRYO CULTURE

A bottleneck to high efficiency transformation in wheat continues to be low plant regeneration rates. In the present study, regeneration rates were improved significantly by creating tissue culture media and culture regimes that simulate the environment *in ovulo*, which nourishes wheat zygotes and young embryos. The *Triticum* embryo culture medium (TEC) includes amino acids, organic acids, and sugars that are found specifically in wheat kernels during embryony. When compared with Murashige and Skoog's basal medium, embryos cultured on TEC medium showed decreased precocious germination, improved callus formation, somatic embryo formation and regeneration from somatic embryos. Further improvement of regeneration frequencies was observed when embryos were cultured at a reduced (2.1 mM) O_2 concentration rather than atmospheric (7.4 mM) O_2 concentration.

Introduction

Regeneration is a major problem in the genetic engineering of wheat (*Triticum aestivum* L.) and other cereal tissues. This paper describes the development of a medium that improves somatic embryo development and techniques to regenerate multiple plants from the initial embryo. Embryo tissue is most easily regenerated. Somatic embryos are generated from immature embryos harvested 12 d after anthesis. Optimal success involves growing the donor plant under a 19 °C day and 16 °C night with a 16 h photoperiod (Hess and Carman 1998).

Classical methods of wheat tissue culture have involved callus induction from different explant tissues followed by organogenesis and plantlet regeneration. O'Hara and Street (1978) tested various wheat explants (nodal and internodal stem segments, wheat embryos, and rachis segments) for callus culture initiation, and concluded that immature wheat embryo tissues were the most competent tissue for callus induction. Embryogenic competence describes the relative ease in which embryogenic cells are produced (Carman and Campbell, 1990). This depends on how far along the developmental pathway meristems are toward organ development, or the degree of cellular differentiation that has already taken place. While shoot meristems are less competent due to their genetic programming for shoot formation produced (Carman and Campbell, 1990), immature embryos are less developed and less differentiated, thus allowing the de-differentiation of embryo cells, followed by re-differentiation into somatic embryos.

Modifications of Murashige and Skoog's (MS) basal medium (1962) have been made to improve induction of embryogenic callus and somatic embryos (Maheshwari et al., 1995). Double strength MS basal medium (DMS) has been used (Ozias-Akins and Vasil, 1982). Fennell et al. (1996) reported that callus initiation and regeneration of plantlets varies depending on the initiation medium. Genotype of the plant and the environment in which it is grown also is critical for high callus induction and regeneration from wheat embryos (Hess and Carman, 1998).

A novel tissue culture medium, designed by Hess and Carman (1993), improves somatic embryogenesis and reduces precocious germination of immature embryos, a process that halts somatic embryogenesis. This medium simulates *in ovulo* minerals, carbohydrates, amino acids and other nutrients (Carman et al., 1993) and hormones (Hess

et al., 2002) from a spring wheat breeding line, PCYT 10. The developing wheat eggs, zygotes, zygotic embryos (Carman, 1989) and somatic embryos (Ehlers et al., 1999; Verdeil et al., 2001) are not attached to parent tissue and acquire nutrients by absorption through the plasma membranes of their cells (Dreyer et al., 1999).

Developing wheat embryos also are exposed to lower O_2 levels in the kernal (Carman 1988), Carman and Campbell (1990) found that a reduction in O_2 concentrations from 7.4 mM (atmospheric) to 2.1 mM (at 1430 m above sea level, Logan, UT) greatly increased the generation of somatic embryos on modified-MS media. These findings prompted the testing of a reduced O_2 culture environment with the *in ovulo* medium.

In this paper we investigated how further modifications to the mineral composition of the *in ovulo* medium and the use of low O_2 tension had an impact on the number of somatic embryos per explant, and number of plants regenerated per cultured explant.

Materials and Methods

Media composition

The medium, termed 100% TEC, used in these experiments was based on the findings of Carman et al. (1996). It consisted of 2.47 g l^{-1} $MgSO_4$, 0.40 g l-1 NH_4NO_3, 0.54 g l-1 NH_2PO_4, 0.18 g l^{-1} NaCl, 1.66 mg l^{-1} KI, 12.4 mg l^{-1} H_3BO_3, 44.6 mg l^{-1} $MnSO_4$ $4H_2O$, 17.2 mg l^{-1} $ZnSO_4$ $7H_2O$, 0.5 mg l^{-1} $NaMO_4$ $2H_2O$, 0.05 mg l^{-1} $CuSO_4$ $7H_2O$, 0.0002 mg l^{-1} $CoCl_2$ $6H_2O$, 1.0 mg l^{-1} nicotinic acid, 1.0 mg l^{-1} pyridoxine HCl, 0.2 mg l^{-1} thiamine HCl, 1341.0 mgl^{-1} malate, 565.0 mg l^{-1} $(NH_4)2HC_6H_5O_7$, 100.0 mg l^{-1} casein hydrolysate, 343.3 mg l^{-1} proline, 348.4 mg l^{-1} arginine, 199.7 mg l^{-1} aspartic acid,

438.3 mg l^{-1} glutamic acid, 225.2 mg l^{-1} asparagine, 365.3 mg l^{-1} glutamine, 1336.4 mg l^{-1} alanine, 2102.0 mg l^{-1} serine, 262.4 mg l^{-1} leucine, 375.4 mg l^{-1} glycine, 180.2 mg l^{-1} methionine, 234.2 mg l^{-1} valine, 292.4 mg l^{-1} lysine, 262.4 mg l^{-1} isoleucine, 165.2 mg l^{-1} phenylalanine, 73.4 mg l^{-1} Fe EDTA, 2.58 g l^{-1} KOH.

The medium was prepared from stock solutions made according to Table 3-1 by adding to 250 mL of distilled and deionized H_2O: 80 ml major mineral stock, 4 ml minor mineral stock, 80 ml organic and amino acid stock, 5 ml vitamin stock, 50 ml iron stock, 14 g myo-inositol, 34 g sucrose, 4.03 g fructose 1,6 diphosphate Ca salt, 7.21 g; maltose, 6.8 ml KOH (5.0 M stock), 5.0 µm 2,4-D and 2.0 µm kinetin. The medium was adjusted to a pH to 5.2 with 1.0 M KOH and/or malate and brought to a volume of 700 mL, warmed to 70 °C and filter- sterilized into a sterile flask. Phytagel (Sigma Chemical Company, St. Louis, MO, USA) (2.0 g) was added to 300 mL distilled and deionized water and autoclaved for 15 min. While at 65 to 70 °C, the Phytagel was thoroughly mixed into the warm nutrient medium and immediately dispensed into Petri dishes (28 ml per dish) under aseptic conditions.

Double (DMS)- and half -strength (HMS) MS media were used for comparison. DMS consisted of double strength MS minerals, normal strength MS vitamins and myo-inositol, 100mg L^{-1} casein hydrolysate, 34 g L^{-1} sucrose, and 1.0 µm 2,4-D and 2.0 µm kinetin. The medium was solidified with 2.0 g L^{-1} Phytagel, and pH adjusted to 5.2. HMS consisted of 1/2 strength MS basal salts, vitamins and myo-inositol, 15 g L^{-1} sucrose, 2.0 g L^{-1} Phytagel, pH 5.75.

Table 3-1. Components of TEC medium broken down into stock solutions.

Stock solution	Medium concentration	Stock concentration
1. Major Mineral:		
	($g\ l^{-1}$)	10X ($g\ l^{-1}$)
$MgSO_4\ 7H_2O$	2.47	24.65
NH_4NO_3	0.400	4.00
KH_2PO_4	0.544	5.44
NaCl	0.175	1.75
2. Minor Mineral:		
	($mg\ l^{-1}$)	200X ($mg\ l^{-1}$)
KI	1.66	332
H_3BO_3	12.4	2480
$MnSO_4\ 4H_2O$	44.6	8920
$ZnSO_4 7H2O$	17.2	3440
$NaMoO_4\ 2H_2O$	0.5	100
$CuSO_4\ 7H_2O$	0.05	10
$CoCl_2\ 6H_2O$	0.0002	0.04
3. Vitamin:		
	($mg\ l^{-1}$)	200X ($mg\ l^{-1}$)
Nicotinic acid	1.0	200
Pyridoxine HCl	1.0	200
Thiamine HCl	0.2	40
4. Organic and amino acid:		
	($mg\ l^{-1}$)	10X ($mg\ l^{-1}$)
Malate	1341	13.41
Ammonium citrate dibasic	5.65	56.55
Casein hydrolysate	100	1.00
Proline	343 3	3 43
Arginine	348.4	3.48
Aspartic acid	199.7	2.00
Glutamic acid	438.3	4.38
Asparagine	225.2	2.25.
Glutamine	365.3	3.65
Alanine	1336.4	13.36
Serine	2102.0	21.02
Leucine	262.4	2.62
Lycine	375.4	3 75
Methionine	180.2	1.80
Valine	234.2	2.34
Lysine	292.4	2.92
Isoleucine	262.4	2.62
Phenylalanine	165.2	1.65
5. Iron:		
	($mg\ l^{-1}$)	2 mM (mg/500 ml)
FeEDTA	73.4	367
6. KOH (5 M):		
	($g\ l^{-1}$)	5 M (g/500 ml)
KOH	2.58	140.28

Tissue culture experiments

Spring wheat PCYT-10 was grown in a greenhouse as described by Carman et al. (1988). Spikes from each plant reaching anthesis (first anther exertion) were tagged by anthesis date, and were removed 12 d past anthesis. Immature embryos were used in two experiments to determine the optimum levels of nutrients in TEC medium and the ideal O_2 level for high frequency somatic embryogenesis. Caryopsis from the excised 12 DPA spikes were removed and surface sterilized in 0.8% (v/v) NaOCl solution for 20 m, followed by three 5 m rinses with sterile ddH_2O.

The first experiment with eight media (1-8) used four concentrations of TEC-based major nutrients at two carbohydrate levels. Sucrose and maltose were used at 75% and 100% TEC medium. The formulations are designated as follows; Medium 1, 50% TEC Nutrients, 75% Carbohydrates; Medium 2, 50% TEC Nutrients, 100% Carbohydrates; Medium 3, 75% TEC Nutrients, 75% Carbohydrates; Medium 4, 75% TEC Nutrients, 100% Carbohydrates; Medium 5, 100% TEC Nutrients, 75% Carbohydrates; Medium 6, 100% TEC Nutrients, 100% Carbohydrates; Medium 7, 125% TEC Nutrients, 75% Carbohydrates; Medium 8, 125% TEC Nutrients, 100% Carbohydrates.

Three replications were performed for each of the eight treatments, with 10 immature embryo explants per treatment. Embryos were placed embryo axis down on medium (to prevent precocious germination) and were cultured in the dark at 25 °C in a chamber flooded with nitrogen to displace O_2. The O_2 level was maintained at 2.1 mM using an O_2 sensor (Seatronics, Hatboro, PA) attached to a datalogger (Campbell Scientific, Logan, UT) that controlled a solenoid valve which flooded the chamber with

N_2 gas when the O_2 level were above the set point. After approximately 28 d, the explants were subcultured to fresh media, and returned to the low O_2 chamber. After 14 additional days, the explants were transferred to HMS medium and placed under fluorescent lighting with a 16 h photoperiod at 7.4 mM O_2 and 25 °C for plantlet regeneration.

Percentages of explants that had precociously germinated were recorded after 21 d. The number of explants with embryogenic callus and the number of somatic embryos per explant (as determined by nodules in the callus that possessed a prominent scutellum-like protrusion) were recorded at 28 d after culture initiation.

The second experiment examined the affect of O_2 concentration (7.4 mM and 2.1 mM). Two media were used: the media producing the best results in the first study and DMS medium at two O_2 levels. In addition to the 2.1 mM chamber described above, a second chamber supplied with normal air at 7.4 mM O_2 was used.

All measurements were subjected to analysis of variance (Sigma Stat 2.0, Jandel Scientific) with a 0.05 level of probability ($p<0.05$).

Results and discussion

The greatest number of somatic embryos and plantlets produced per explant was obtained with 75% TEC medium at 100 % carbohydrate levels and low O_2 tension (medium 4, Figure 3-1). While other medium and carbohydrate combinations yield closer ratios of somatic embryos to regenerated plantlets (medium 2), our goal was to obtain the maximum number of somatic embryos and maximum number of regenerated plantlets. The numbers of somatic embryos produced on both medium 3 and 5 were near that of medium 4, but there were at least 50% fewer regenerated plantlets. Medium 2 and 8 had

produced the fewest somatic embryos, due to low and high concentrations of TEC nutrients respectively. TEC medium 4 was subsequently used for experiment two to compare TEC and DMS media.

TEC medium 4 produced better results than the DMS media in the second study (Figure 3-2). Immature wheat embryos cultured on TEC medium 4 at both 7.4 mM and 2.1 mM O_2 produced significantly greater percentages of embryogenic callus ($P=0.01$) and somatic embryos ($P<0.001$), significantly more somatic embryos per explant ($P<0.001$), but significantly fewer precociously germinating explants ($P>0.001$) than did immature embryos cultured on DMS medium (Figure 3-2). Embyros grown on TEC medium at 2.1 mM O_2 produced more callus, a greater number of somatic embryos, and a greater number of somatic embryos per explant than were produced on embryos grown on TEC medium and cultured at 7.4 mM O_2 (Figure 3-2). Reduced O_2 tensions also reduced the precocious germination of embryos grown on TEC medium.

Differences in composition of DMS and TEC media include: 1) most nitrogen in TEC medium is from amino acids, while less than 1% of nitrogen comes from amino acids in DMS, 2) PO_4 levels in TEC medium are nearly seven times higher, 3) TEC medium has 2-3 times more magnesium and sodium than DMS, 4) malate and citrate, common in the embryo sac fluids (Murray, 1988), are present in TEC but not in DMS medium, 5) there is 14 times more myo-inositol in TEC, 6) TEC medium has nearly two times as much sucrose. These differences are summarized in Table 3-2.

Lowering of O_2 concentrations from 7.4 to 2.1 mM O_2 improved responses of the immature embryo explants cultured on both TEC and DMS media. There was a twofold increase in the percentage of embryogenic callus and somatic embryos produced by

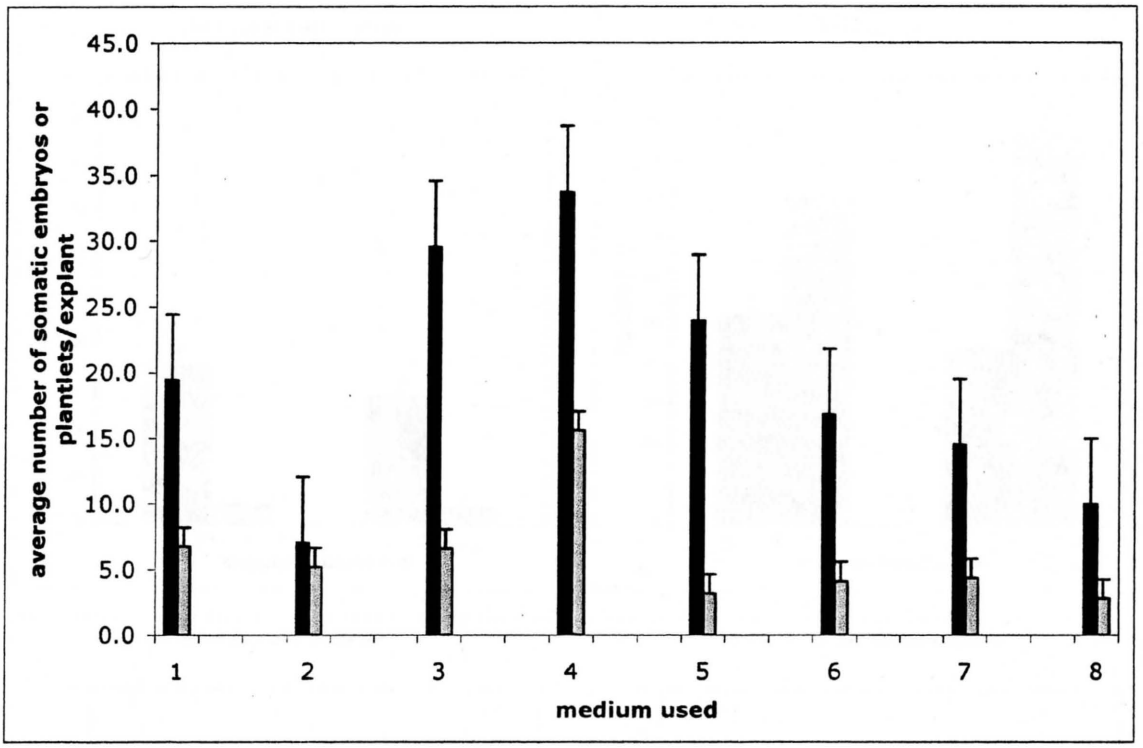

Figure 3-1. Comparison of TEC medium major nutrient and carbohydrate levels on somatic embryo and plantlet formation at 2.1 mM O_2. Medium 1, 100% TEC, 75% Carbohydrate; Medium 2, 50% TEC, 100% Carbohydrate; Medium 3, 75% TEC, 75% Carbohydrate; Medium 4, 75% TEC, 100% Carbohydrate; Medium 5, 100% TEC, 75% Carbohydrate; Medium 6, 100% TEC, 100%, Carbohydrate; Medium 7, 125% TEC, 75% Carbohydrate; Medium 8, 125% TEC, 100% Carbohydrate. ■ Somatic Embryos; ▩ Plantlets. See text for details on TEC medium composition. Error bars equal standard error from the mean.

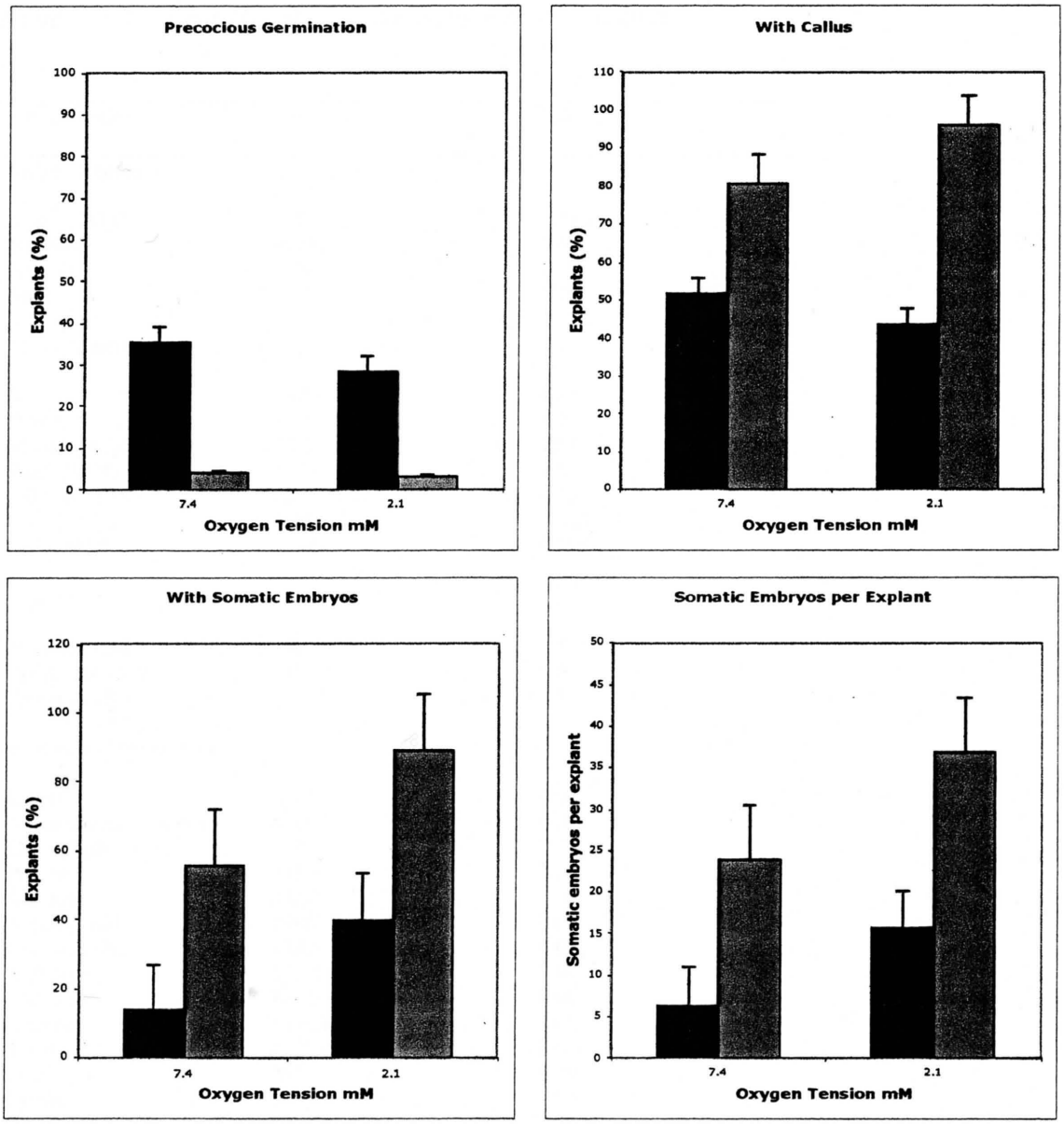

Figure 3-2. Immature embryos from wheat PCYT 10 were excised at 12 DPA and grown on either TEC or MS medium at either 2.1 mM or 7.4 mM O_2 concentration. Precocious germination (# germinated embryos)/(total embryos). With callus (# explants producing embryogenic callus)/(total embryos). With somatic embryos (# somatic embryos)/(total embryos). Number of somatic embryos per explant at 7.4mM and 2.1 mM O2 levels after 28 d on DMS or TEC media. Somatic embryos derived from PCYT 10 immature embryos excised at 12 DPA. Error bars represent standard error. ■ -DMS, ■ -TEC. Error bars equal standard error from the mean.

Table 3-2. Comparison of TEC medium and MS medium.

Stock solution	TEC	MS
Major Mineral:		
$MgSO_4$ $7H_2O$	2.47	0.18
NH_4NO_3	0.400	1.6
KH_2PO_4	0.544	0.17
NaCl	0.175	0.00
Minor Mineral:		
KI	1.66	0.83
H_3BO_3	12.4	6.2
$MnSsO_4$ $4H_2O$	44.6	22.3
$ZnSO_4$7H2O	17.2	8.6
$NaMoO_4$ $2H_2O$	0.5	0.25
$CuSO_4$ $7H_2O$	0.05	0.025
$CoCl_2$ $6H_2O$	0.0002	0.025
Vitamin:		
Nicotinic acid	1.0	0.5
Pyridoxine HCl	1.0	0.5
Thiamine HCl	0.2	0.1
Organic and amino acid:		
Malate	1341	0.00
Ammonium citrate dibasic	5.65	0.00
Casein hydrolysate	100	0.00
Proline	343 3	0.00
Arginine	348.4	0.00
Aspartic acid	199.7	0.00
Glutamic acid	438.3	0.00
Asparagine	225.2	0.00
Glutamine	365.3	0.00
Alanine	1336.4	0.00
Serine	2102.0	0.00
Leucine	262.4	0.00
Lycine	375.4	0.00
Methionine	180.2	0.00
Valine	234.2	0.00
Lysine	292.4	0.00
Isoleucine	262.4	0.00
Phenylalanine	165.2	0.00
Iron:		
FeEDTA	73.4	37.26
KOH (5 M):		
KOH	2.58	

explants cultured on TEC medium, and far less precocious germination of the immature embryos explants (Figure 3-2). The lower O_2 tensions may increase abscisic acid levels, thus, preventing precocious germination *in situ* and *in vitro* (Hess and Carman, 1993). It is speculated that precocious germination has a negative effect on the initiation of embryogenic callus and somatic embryo formation because the developing embryo is locked into the germination pathway.

In this study, an optimized medium, TEC medium 4 (75% TEC medium and 100% carbohydrate) coupled with a 2.1 mM O_2 culture environment not only out performed all other TEC medium combinations including *in ovulo* levels, but also the standard DMS medium.

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CHAPTER 4

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION OF WHEAT (*TRITICUM AESTIVUM* L.) USING ANTIOXIDANTS AND HYPOXIC CULTURE ENVIRONMENTS

High-efficiency *Agrobacterium*-mediated transformation of wheat is limited by tissue necrosis following co-cultivation, and by poor plant regeneration. In this study we investigate the effects of reducing necrosis by adding antioxidant compounds and increasing regeneration by manipulating culture media and O_2 tensions. Wheat embryos were cultured at 12 d past anthesis (DPA), co-cultivated with *Agrobacterium tumefaciens* strains WAg11 or EHA101, incubated on a novel plant tissue culture medium containing the antioxidant compounds catalase, cysteine and ascorbic acid, and exposed to lowered O_2 concentrations (2.1 mM O_2 vs. atmospheric O_2 concentration 7.4 mM O_2). Genetic transformation was documented in 6.0% of regenerated *A. tumefaciens* WAg11 exposed wheat using the firefly luciferase (*luc*) reporter system.

Introduction

Agrobacterium-mediated DNA transfer into plants is used for plant genetic engineering (Gelvin, 2003). However, genetic transformation of wheat and other monocot species is difficult using *Agrobacterium tumefaciens* as a vector. Typical transformation efficiencies (percentage of explants exposed to *Agrobacterium* showing transformation) range from 30% in rice (Enríquez-Obregón et al., 1999) to 1.5% in wheat (Carman et al., 1993; Cheng et al., 1997), while transformation efficiencies using biolistics range from 0.01%- 1% (Smith and Hood, 1995). This is compared to 60-86% in

tobacco (Bidney et al., 1992). *Agrobacterium* species colonize and may form small tumors in such monocots as *Allium*, *Asparagus*, *Hordeum*, and *Triticum* (Connor and Dommisse, 1992).

Potential difficulties for transformation include limited plant cell growth and regeneration after exposure to *Agrobacterium* (Perl et al., 1996), and integration of the T-DNA into the plant genome (Nam et al., 1997). Low regeneration may be related to the possibility that monocotyledonous cells lose the ability to de-differentiate early in development (Graves et al., 1988). They suggested that this trait affects interactions with microbes. Whether integration of T-DNA into the wheat genome is a problem has not been determined. Raineri et al (1990) and Gould et al. (1991) demonstrated the integration of T-DNA into rice and maize respectively, showing that this was not an impediment to transformation in these monocots.

A serious impediment to culture of healthy transformed tissues is the plant cell necrosis induced in wheat by *Agrobacterium* challenge (Hansen, 2000; Parrott et al., 1997). Necrosis is induced in other plant tissues that are difficult to transform: grape, maize, and the woody species aspen and poplar (Perl et al., 1996; Hansen, 2000; De Block, 1990.). A reduction in cell death by the inclusion of other antioxidants is correlated with improved transformation efficiency in Japonica rice, sugarcane and sorghum (Enríquez-Obergón et al., 1997, 1999; Zhao et al., 2000, Hondred et al., 2000). Ascorbic acid, cysteine, and silver nitrate were used as antioxidants and polyvinyl-polypyrrolidone (PVPP) was included to sorb phenolic compounds. The increase in transformation efficiencies in these plants correlates with reduction in browning and necrosis of plant tissues after exposure to *Agrobacterium*. We also found that a reduction

in O_2 concentration from normal (7.4 mM at 1430 m above sea level, Logan, UT) to the 2.1 mM ovular level (Carman, 1995) lessened the degree of necrosis after *Agrobacterium* challenge (see Chapter 2). This finding is interesting because reduced O_2 levels also improve other features that are important in maximizing wheat transformation by increasing callus and somatic embryo formation and decreasing precocious germination of embryos (Carman, 1995).

The objective of this research was to examine the effect of combining the antioxidant process with modified medium and O_2 tension on wheat transformation and regeneration. Because it has been suggested the H_2O_2 orchestrates the hypersensitive response (HR) in plants (Lamb and Dixon, 1997) and we demonstrate that in wheat, plant cell death is associated with hydrogen peroxide formation (see Chapter 2), we included catalase in an attempt to remove the hydrogen peroxide generated from *Agrobacterium* challenge. Similarly, we included ascorbic acid and cysteine in a effort to further reduce H_2O_2, because plant viability in rice exposed to *Agrobacterium* was increased 20 and 40%, respectively, with these compounds (Enríquez-Obergón et al., 1999).

Materials and Methods

Strains and T-DNA plasmid

Two binary *Agrobacterium tumefaciens* vectors were used in these experiments. Both strains share the common ancestor *A. tumefaciens* A136 (Sciaky et al., 1978). The first strain, *Agrobacterium tumefaciens* EHA101 (Hood et al., 1986), is a derivative of A281 (Sciaky et al., 1978) containing the Ti plasmid pEH101. The second strain, *Agrobacterium tumefaciens* WAg11 (Barnes, 1990), is a disarmed derivative of A208

(Sciaky et al., 1978). WAg11 contains the Ti plasmid pTiunT92A, a disarmed Ti T37 plasmid containing tandem ampicillin resistance genes in place of the T-DNA region (Barnes, 1990). They were grown on Luria broth (LB) medium (10.0 g l^{-1} tryptone, 5.0 g l^{-1} yeast extract, 10.0 g l^{-1} NaCl, pH 7) or low phosphate AB glucose medium (Kunoh et al., 1983; Lamb and Dixon, 1997) modified with 20.0 mM MES, 2.0 mM phosphate buffer (pH 7.0), 2.0 g l^{-1} L-glutamate, and lacking yeast extract. Stocks of the bacterial isolates were made from a suspension of an overnight bacterial culture grown for 16 h at 31 °C, suspended in 15% sterilized glycerol and frozen at –80 °C.

The helper T-DNA plasmid pWB146 (kindly supplied by Dr. Wayne Barnes) was used as part of the binary plasmid system in both WAg11 and EHA101. Plasmid pWB146 consists of the following inserted into plasmid pRK252: nopaline synthase promotor, CaMV1841 promoter, firefly luciferase cDNA (*luc*), NPTII gene (kanamycin resistance), tomato protease inhibitor I (TomPI), and NOS 3' DNA (Barnes, 1990). This kanamycin resistance gene confers resistance to the transformed plants only (not the bacteria) and was used to prescreen transformed embryos, while the firefly luciferase (*luc*) gene was used as a reporter, allowing detection of light in transformed plants.

Electroporation of T-DNA plasmid into
A. tumefaciens WAg11 and EHA101

Agrobacterium EHA101 or WAg11 cells were grown in LB broth to OD_{600} = 1.0, harvested by centrifugation, resuspended in 3 ml 10% glycerol, aliquoted in 0.2 ml aliquots and frozen. Plasmid pWB146 (200 ng) was added to the thawed cells, and 40 µl of this mixture was transferred to a 2 mm gapped stainless steel electrode cuvette. A 20 ms 2.5 kv/cm, 50 µF capacitor electrical pulse was provided to the suspended cells using

a Promega X-Cell 450 electroporator (Madison, WI, USA). After electroporation, bacterial cells were placed into 0.8 ml SOC medium (20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 mM NaCl, 2.5 KCl mM. 10 mM $MgCl_2$, 20 mM $MgSO_4$ 20 mM glucose, pH to 7.0) and incubated at 28 °C for 1 h, spread on LB tet/kan plates (10 μg ml^{-1} tetracycline, 20 μg ml^{-1} kanamycin) and incubated for 24 h at 28 °C to select for transformed bacteria containing the pWB146 plasmid (Mattanovich et al., 1989). Tetracycline and kanamycin resistance genes resides outside the T-DNA region on the pWB146 plasmid for selection of bacterial colonies containing the electroporated plasmid.

Wheat tissue culture

Spring wheat PCYT 10 embryos were obtained from whole immature embryos removed under sterile conditions from kernels 12 d past anthesis (DPA) (Carman, 1995). Embryos were cultured for 3 d in the dark at 25 °C, embryo axis down (to prevent precocious germination), on *Triticum* Embryo Culture (TEC) medium pH 5 (Chapter 3) supplemented with 2.0 μM kinetin and 5.0 μM 2,4-D, 2.0 g l^{-1} and solidified with Phytagel. For comparison, Murashige and Skoog (1962) basal medium was supplemented similarly. The embryos which were grown at low O_2 concentration (2.1mM) were placed in a chamber where the O_2 level was maintained at using an O_2 sensor (Seatronics, Hatboro, PA). This was attached to a datalogger (Campbell Scientific, Logan, UT), which controlled a valve allowing the chamber to be flooded with N_2 gas when the O_2 level rose above 2.1 mM O_2. The embryos grown at 7.4 mM O_2 were placed in a similar chamber without reduction of O_2 levels (Chapter 3).

Wheat transformation

Wheat embryos grown as described above (3 d in culture) were exposed to *Agrobacterium tumefaciens* EHA101 or WAg11. Four replications each with 250 embryos were carried out. The bacteria were grown to an OD_{600}=1.0, ($1x10^8$ colony forming units/ml) in 25 ml low phosphate AB glucose medium (Kunoh et al., 1983; Lamb and Dixon, 1997) modified with 20.0 mM MES, 2.0 mM phosphate buffer (pH 7.0), 2.0 g l^{-1} L-glutamate, and lacking yeast extract. The bacteria were pelleted, supernatant discarded, and the bacteria suspended in 0.3 vol (7.5 ml) TEC or MS liquid medium (depending on the medium used for explant growth) lacking kinetin and 2,4,D but supplemented with 100 μM acetosyringone and an antioxidant mixture (AO) of 20 mg L^{-1} ascorbic acid and 40 mg L^{-1} cysteine. Embryos were placed in the bacterial suspension for 2 min, removed, and blotted dry on sterile filter paper. The inoculated embryos were transferred to co-cultivation medium (solid TEC or MS medium as described above, supplemented with 100 μM acetosyringone, and either of two antioxidant regimes; 30 mg ml^{-1} catalase (bovine liver, ~40000 U mg^{-1}, Sigma Chemical Company, St. Louis, MO, USA), or 20 mg l^{-1} ascorbic acid and 40 mg l^{-1} cysteine (Enriques-Obregon et al., 1997), or no antioxidant compounds) and incubated at 25 °C in the dark at either 2.1 mM or 7.4 mM O_2. Co-cultivation was carried out for 48 h, at which time embryos were removed from co-cultivation medium to liquid TEC or MS medium containing antioxidants (20 mg L^{-1} ascorbic acid, 40 mg l^{-1} cysteine) and 10 mg ml^{-1} of the antibiotic Augmenten (GalaxoSmithKlein, Raleigh-Durham, NC, USA), for 5 m with gentle agitation to kill *Agrobacterium* cells. The embryos were then transferred to solid TEC or MS medium containing 30 mg ml^{-1} catalase (TEC+CAT, MS+CAT), 20 mg l^{-1}

ascorbic acid and 40 mg l^{-1} cysteine (TEC+AO, MS+AO), or no antioxidant compounds (TEC, MS), 250 μg ml^{-1} kanamycin (for transgenic plantlet selection) and 10 mg ml^{-1} Augmenten. To summarize the treatments, the wheat embryos were exposed to *A. tumefaciens* WAg11 or EHA101 and grown on TEC, TEC+CAT ,TEC+AO, DMS, DMS+CAT or DMS +AO at either 7.4 mM O_2 or 2.1 mM O_2.

Detection of luciferase in transformed wheat plants

Luciferase activity in transformed wheat embryos or regenerated plants was assayed as described in Ausubel et al. (1992). Embryos growing on medium containing kanamycin were washed in Triton/glycylglycine lysis buffer (1% v/v Triton X-100, 25 mM glycylglycine, 15 mM magnesium sulfate, 4 mM EGTA, 1 mM DTT) to disperse clumped tissues. Cells (100 μl) were transferred to a reaction mixture containing 350 μl of luciferin assay buffer (25 mM glycylglycine; 15 mM potassium phosphate, pH 7.8; 15 mM magnesium sulfate; 4 mM EGTA; 2 mM ATP; 1 mM DTT). Luciferin substrate, 200 μl, (1mM D-luciferin; 25 mM glycylglycine; 10 mM diluted to 200 μM using 25 mM glycylglycine immediately prior to use) was added and light emission recorded in a luminometer (Lumat, Berthold Technologies). A control, lacking *Agrobacterium*-exposed cells, was used to obtain background luminescence levels.

Transformation of wheat cells was verified by southern blot analysis to detect the *luc* gene. Wheat DNA was extracted using a MasterPure Plant Leaf DNA Extraction Kit (Epicentre Technologies, Madison, WI, USA). Bacterial DNA was extracted using a Perfect Plasmid miniprep kit (Eppendorf Scientific, Westbury, NY, USA). All DNA was digested using *Eco*R I (New England BioLabs Beverly, MA, USA), using the procedures

outlined by Hoisington et al. (1994). Each sample DNA (10 μg) was loaded onto a 0.7% agarose gel (SeaKem LE, BioWhittaker Molecular Applications Rockland, ME, USA) and run at 100 mA for 10 m, then at 12 mA for 4 h at 100 V. As a positive control for the chemiluminescent detection kit, pre-biotinylated labeled markers were added.

DNA transfer to nylon membranes (Millipore Immobilon-S Neutral Bedford, MA, USA) was accomplished as in Brown (1987a, b). The probe used was a 30 base segment from the firefly luciferase gene (*luc*): 5' GCGCCCGCGAACGACATTTATAATGAA CGT 3'. This sequence, synthesized by Operon Technologies (Alameda, CA, USA), was labeled 5' and 3' with biotin for chemiluminescent detection. Hybridization of the probe to the immobilized DNA was carried out as outlined in the NeBlot Phototope Kit instruction manual (New England BioLabs, Beverly, MA, USA). This hybridization procedure was optimized for use with the Phototope-Star detection kit (New England BioLabs, Beverly, MA, USA), which was used to detect the hybridized probe. Non-*Agrobacterium* exposed wheat was used as a control, and was treated as described above.

Statistical analysis

All data were subjected to three way analysis of variance (SigmaStat 2.0, Jandel Scientific) and considered significant at a p-value < 0.05 (tables shown in Appendix B).

Results

Wheat tissue culture and regeneration of plantlets

Approximately 12,200 somatic embryos formed on 960 embryo explants incubated at 2.1 mM O_2, from which 1144 plantlets regenerated. This compared to 7960

somatic embryos from 960 embryo explants incubated at 7.4 mM O_2 from which 631 plantlets were regenerated (Table 4-1A). Reduction in O_2 concentrations from 7.4 mM to 2.1 mM significantly ($P<0.001$) increased in somatic embryogenesis and plant regeneration on all media used. The use of TEC medium shower significant improvement ($P<0.001$) in somatic embryo formation and plant regeneration when compared to DMS. Addition of anti-oxidant compounds to both TEC and DMS media significantly ($P<0.001$) improved the formation of wheat somatic embryos and the regeneration of plantlets. Explants exposed to WAg11 and grown in 2.1 mM O_2 on TEC medium supplemented with the anti-oxidant mixture (TEC+AO) produced significantly ($P<0.001$) greater numbers of somatic embryos and regenerated plantlets than did any other treatment (Figure 4-1). A representative *Agrobacterium*-inoculated wheat explant is shown in Figure 4-2. Various stages of development are evident, including somatic embryos (black arrow) and germinating plantlets (white arrow).

Transformation of wheat plants

There were a total of 631 regenerated plantlets and five transformants formed on 480 explants grown on all media combinations at 7.4 mM O_2. At 2.1 mM O_2, there were a total of 1144 regenerated plantlets and 24 transformants formed on 480 explants, 15 from exposure to WAg11, and nine from exposure to EHA101. Percent transformation based on both percent of transformed plants per explant (EXP) and percent transformed plants per regenerated plants (REG) is shown in Table 4-1B. Lowering O_2 tensions significantly ($P<0.001$) increased the number of regenerated plantlets and subsequently

Table 4-1. A. Number of somatic embryos and regenerated plantlets formed for each treatment. Data shown represent six media treatments, 10 explants per treatment, two O_2 levels, two bacterial strains, and four replications of the experiment. SE, somatic embryos. REG, regenerated plantlets. B. Transformation efficiencies for *A. tumefaciens* WAg11 and EHA101 on wheat somatic embryos. Data shown represent six media treatments, 10 explants per treatment, two O_2 levels, two bacterial strains, and four replications of the experiment. EXP, % transformed plants per explants. REG, % transformed plants per regenerated plantlets.

A

	7.4 mM O_2				2.1 mM O_2			
	WAg11		EHA101		WAg11		EHA101	
Medium	SE	REG	SE	REG	SE	REG	SE	REG
TEC	822	60	794	58	1274	117	1282	118
TEC+CAT	838	69	810	66	1372	129	1338	126
TEC+AO	952	84	946	83	1550	150	1508	146
2xMS	382	25	500	33	530	48	564	51
2x MS+CAT	432	34	488	38	602	55	664	61
2xMS+AO	476	39	520	42	712	67	804	76

B

	% Transformation							
	7.4 mM O_2				2.1 mM O_2			
	WAg11		EHA101		WAg11		EHA101	
Medium	EXP	REG	EXP	REG	EXP	REG	EXP	REG
TEC	1	1	1	1	5	2	3	1
TEC+CAT	3	1	0	0	3	1	9	3
TEC+AO	4	2	0	0	23	6	5	1
2xMS	0	0	0	0	0	0	0	0
2x MS+CAT	0	0	0	0	3	2	0	0
2xMS+AO	0	0	3	2	5	3	6	3

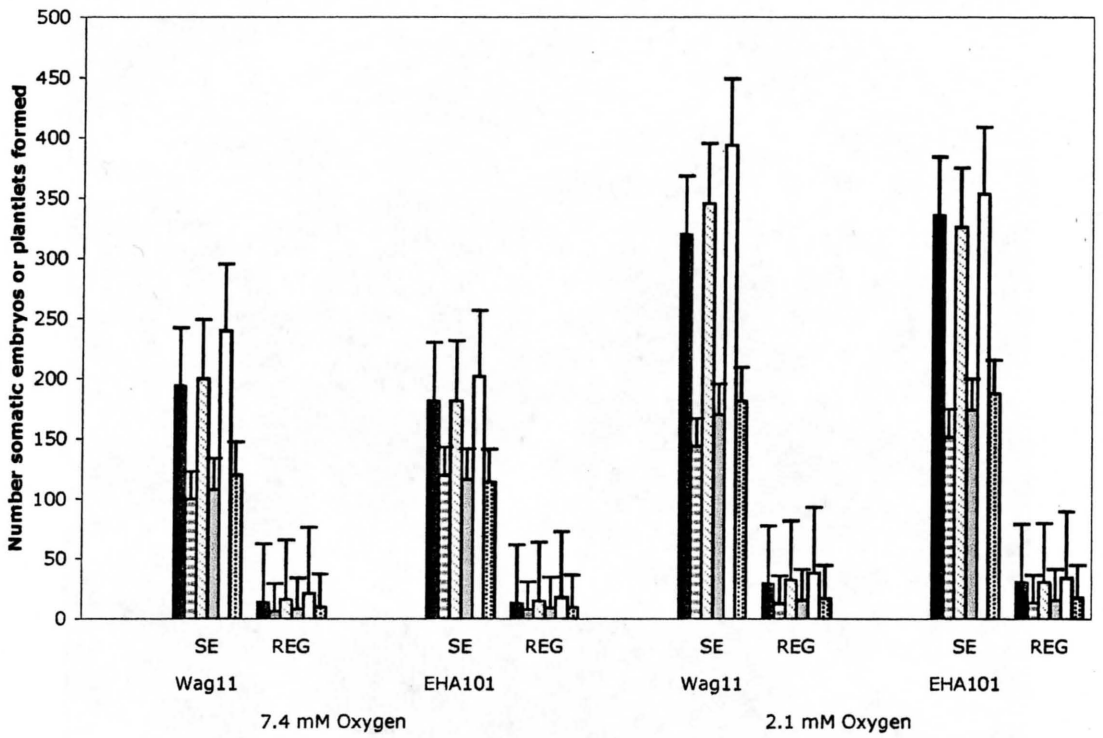

Figure. 4-1. Comparison of O_2 level, bacterial exposure, and medium type on production of somatic embryos and regenerated plantlets. ■ TEC medium. ▤ 2x MS; double strength MS medium. ▨ TEC+CAT; TEC medium with the addition of 30 mg ml^{-1} catalase. ■ 2x MS+CAT; double strength MS medium with the addition of 30 mg ml-1 catalase. □ TEC+AO; TEC medium with the addition of 20 mg l^{-1} ascorbic acid and 40 mg l^{-1} cysteine. ⊡ 2x MS+AO; double strength MS medium with the addition of 20 mg l^{-1} ascorbic acid and 40 mg l^{-1} cysteine. Somatic emb; number of somatic embryos per 20 explants. Regenerants; number of regenerated plantlets per 20 explants. Figure representative of four replications of experiment. Error bars equal standard error from the mean.

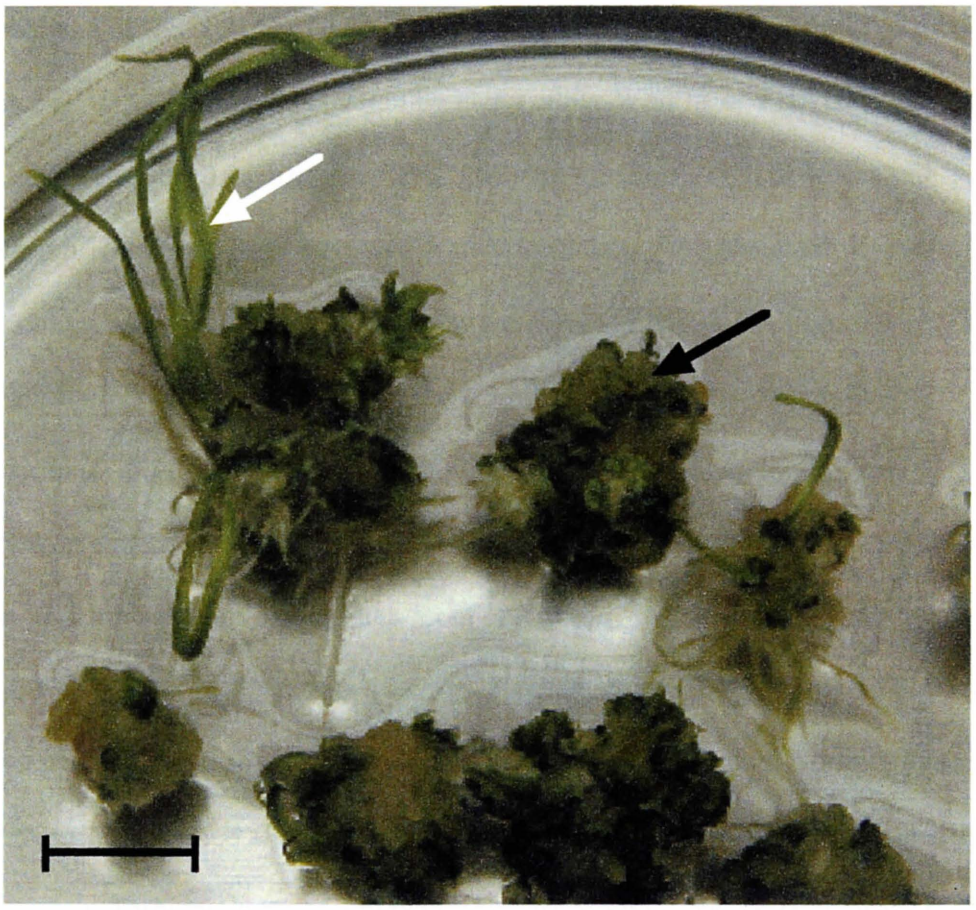

Figure 4-2. Agrobacterium exposed wheat embryos 28 d after co-cultivation. Note green somatic embryos (black arrow) and germinating plantlets (white arrow). Scale bar = 1 cm.

the percentage of transformed plants (Figure 4-3). However, there was no significant difference ($P=0.137$) in transformation by using either WAg11 or EHA101. The use of TEC medium increased significantly ($P<0.001$) the transformation of wheat embryos. Further significant ($P<0.001$) increases in transformation were observed when anti-oxidant compounds were added to the culture medium (Table 4-1B; Figure 4-4). The combination of TEC+AO medium and the 2.1 mM O_2 concentration resulted in significantly ($P<0.001$) higher transformation and regeneration rates (Table 4-1B; Figure 4-4) when compared to embryos grown at atmospheric O_2 conditions (7.4 mM O_2) on MS medium, regardless of antioxidant treatment or *Agrobacterium* strain (Figure 4-3, Table 4-1B). All 29 regenerated plantlets growing on media containing kanamycin displayed luminescence ranging from 1261 rlu to 20435 rlu above background per transformed explant. Non-transformed and control plantlets displayed counts at background level. DNA extracted from the luminescent plants hybridized with the *luc* gene probe (Figure 4-4), while non-*Agrobacterium* exposed control plant DNA did not hybridize with the probe (Figure 4-4).

Discussion

Transformation of wheat at low efficiency (0.01-1.5%) has been well documented previously (e.g., Carman et al., 1993; Cheng et al., 1997; Mooney et al., 1991; Harvey et al., 1999). Many methods have attempted to improve efficiency of T-DNA transfer into plants, including electroporation (Mattanovich et al., 1989) and sonication (Trick and Finer, 1998) of plant cells, presumably to make cell membranes more porous and

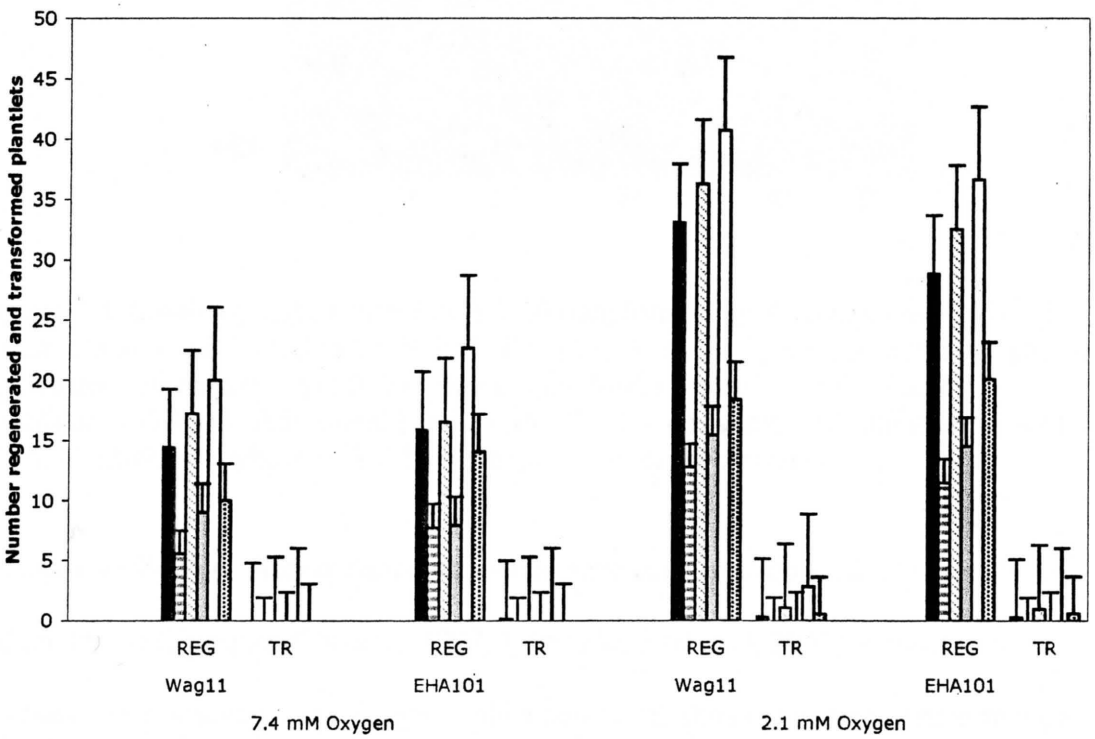

Figure. 4-3. Comparison of O_2 level, bacterial exposure, and medium type on production of regenerated and transformed plantlets. ■ TEC medium. ▤ 2x MS; double strength MS medium. ▧ TEC+CAT; TEC medium with the addition of 30 mg ml^{-1} catalase. ■ 2x MS+CAT; double strength MS medium with the addition of 30 mg ml^{-1} catalase. □ TEC+AO; TEC medium with the addition of 20 mg l^{-1} ascorbic acid and 40 mg l^{-1} cysteine. ⊡ 2x MS+AO; double strength MS medium with the addition of 20 mg l^{-1} ascorbic acid and 40 mg l^{-1} cysteine. REG; number of regenerated plantlets per 20 explants. TR; number of transformed plantlets per 20 explants. Figure representative of four replications of experiment. Error bars equal standard error from the mean.

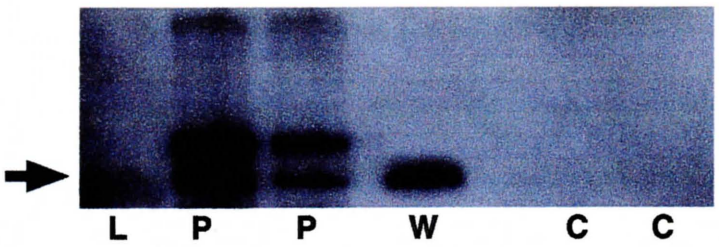

Figure 4-4. Southern blot of wheat PCYT 10 transformed by *A. tumefaciens* WAg11, digested with *Eco*R I. L, ladder; P, PCYT 10; W, WAg11; C, control. Band marked by black arrow corresponds to the *luc* gene in transformed wheat PCYT 10 and *A. tumefaciens* WAg11. Additional bands in PCYT 10 lanes show multiple insertions of T-DNA. Controls are wheat PCYT 10 not exposed to *Agrobacterium*.

receptive to T-DNA. Antioxidant compounds have been incorporated into plant tissue culture media (Enriques-Obregon, 1997, 1999; Hondred et al, 2000) to limit *Agrobacterium*-induced browning and plant cell death, thus increasing transformation efficiencies. Previously, we found (see Chapter 2) a reduction in plant cell death due to *Agrobacterium* exposure when O_2 concentrations were reduced to *in ovulo* (Carman, 1995) levels (2.1 mM O_2). Antioxidant treatments further reduced the negative effects of *Agrobacterium* on plant cells. Catalase, ascorbic acid and cysteine were used throughout the transformation process, not just as additives in the tissue culture medium. Testing these compounds with normal and low O_2 tensions showed an increase in somatic embryos, regenerated plantlets and transformed plants (Figures 4-1, 4-4). The significant ($P<0.001$) correlation between O_2 level and medium was demonstrated with the combination of TEC+AO and 2.1 mM O_2, which increased about two-fold the level of transformation in wheat above previously published levels (Cheng et al., 1997; Mooney et al., 1991; Harvey et al., 1999), thus overcoming the limitations that cellular necrosis and poor plant regeneration impose on *Agrobacterium*-mediated transformation of wheat.

While our findings report low transformation efficiencies that are less than that of dicots (60-68% in tobacco), there are areas of further research that could increase wheat transformation levels even further. These include experimentation with different strains of *A. tumefaciens* and with *A. rhizogenes* to determine whether they vary in induction of plant necrosis and potential induction of plant defenses, and are more efficient in the transfer of T-DNA into wheat. It may also be possible to test different types of plasmid constructs (binary and co-integrated) to determine optimal transfer of T-DNA. Different promoters in the Ti helper plasmid constructs may also increase gene expression in the transformed plant cell. Further information gained from this and other proposed research, in combination of our current techniques in wheat culture and transformation, may result in the high efficiency of wheat transformation in the near future.

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CHAPTER 5

SUMMARY

The general premise of this research was to determine why *Agrobacterium* interactions with wheat resulted in very low frequency transformation. The objectives were to determine whether:

1) plant defense mechanisms were activated by *Agrobacterium;*

2) factors that reduce plant defense mechanisms increased transformation efficiencies;

3) an integration of cutting-edge plant cell culturing techniques improved the regeneration of the transformed plants.

My research has revealed *in vitro* manipulation of chemical and environmental conditions that increased successful transformation of wheat using *Agrobacterium.* Significant and novel findings of my research are as follows.

Chapter 2

1) bacterial growth Is not inhibited by wheat factors;

2) wheat roots are effectively colonized by *Agrobacterium tumefaciens* and *A. rhizogenes;*

3) *A. tumefaciens* and *A. rhizogenes* elicit plant cell death in wheat involving H_2O_2 evolution and an increase in ferulic acid in the wheat cell walls;

4) *Agrobacterium*-induced plant cell death is reduced using lowered O_2 environments.

My first hypothesis that wheat transformation was limited by the inability of *Agrobacterium* cells to grow in the presence of wheat cells was refuted. My studies showed that *Agrobacterium* was not impeded in growth by the wheat tissues. *Agrobacterium* cells colonized wheat root surfaces to levels (3.0×10^7 cfu/root, +/- 1.0×10^7) comparable with those of saprophytic root colonizers (Buell and Anderson, 1992; Miller et al., 2001). I found no evidence that chemicals, such as DIMBOA that inhibits *Agrobacterium* growth in maize, would inhibit *Agrobacterium* growth in wheat. Growth of *Agrobacterium* cells was not impaired upon wounding wheat tissues, conditions that would cause dicotyledonous plants to produce *vir* gene-activation compounds (Gelvin, 2003) but also possible antimicrobial chemicals. In liquid culture, with wounded wheat tissues, the bacteria thrived (Figure 2-1, Chapter 2).

My studies supported hypothesis two: wheat responds with defense-like reactions to *Agrobacterium*. Wheat cells in contact with *Agrobacterium* exhibited changes typical of resistance events, such as necrosis, browning, and cell death. Elevated levels of H_2O_2, altered cell wall composition and higher levels of cellular necrosis and browning in wheat tissues were observed when inoculated with the either the *A. tumefaciens* or the *A. rhizogenes*. Immature embryos, as well as root tissues were affected. Roots would be the normally challenged tissues in nature. The response in embryos is significant because these are the best tissues for plant regeneration in wheat (Carman and Campbell, 1990; O'Hara and Street, 1978) and for transformation (Smith and Hood, 1995).

These defense responses observed are characteristic of the hypersensitive response observed in incompatible microbial challenges of mono and dicotyledons (Lamb and Dixon, 1997). However, the onset of cell death and increased H_2O_2 formation proceeded simultaneously upon challenge of wheat tissues with either *A. tumefaciens* or *A. rhizogenes* (Figures 2-2, 2-5, Chapter 2), which was distinct from that reported for the hypersensitive response in dicotyledons (Lamb and Dixon, 1997) where cell death occurred after the initial burst of H_2O_2. In my study with wheat, *P. syringae* pv. *syringae* B728a also caused early, simultaneous cell death with H_2O_2 evolution (Figures 2-2, 2-5, Chapter 2). I speculate that the trigger for cell death in wheat may involve a mechanism different from that characterized for the hypersensitive response in race-cultivar interactions (Delledonne et al., 1998). The mechanism remains unclear.

Race-cultivar interactions rely on a product from a specific avirulence gene from the pathogen, transported into the host cell by a specific transfer system from the bacterial cell, to be detected by a resistance gene mechanism in the host, and this detection event triggers apoptosis (Lamb and Dixon, 1997). We found that wheat cell death is caused by factors in *Agrobacterium* culture filtrates and these effects resembled those elicited by extracellular harpins that are produced by certain plant pathogenic bacteria that lack race-cultivar specificity properties (Charkowski et al., 1998).

Because elevated levels of H_2O_2 were induced in wheat cells by *Agrobacterium* exposure, I speculated that changes, specifically phenolic modification such as lignification, in wheat cell walls induced by *Agrobacterium* exposure might be limiting transformation. When exposed to fungal pathogens lignification of wheat cell walls limits ingress of fungal pathogens into the host cell (Hammerschmidt, 1984; Ride et al., 1989).

However, there was no evidence of lignification or callose formation in response to bacterial challenge. Increased autofluorescence was observed in the *Agrobacterium*-challenged wheat cell walls where cell death was occurring and could be related to increased ferulic acid deposition in challenged epidermal cells (Figure 2-4, Chapter 2). Whether such modifications to the wheat cell wall limits the transfer of genetic material is unknown at the present. It is possible that increased levels of H_2O_2 may cause cross linking of cell wall polymers such as lignin, causing a decrease in permeability due to reduction in cell wall pore size (Carpita and Gibeaut, 1993).

My findings agree with other studies that link the induction of plant cell death in other monocots and grape that are difficult to transform by *Agrobacterium* strains with suboptimal transformation efficiency. Reduction of the level of induced cell death and enhanced transformation efficiencies was achieved by expression of the cell death regulator genes *p35* and *iap* in maize (Hansen, 2000). My observation of browning in the *Agrobacterium*-wheat interaction was similar to that observed in grape (Perl et al., 1996), where a reduction in necrogenesis by inclusion of antioxidants permitted the grape to be transformed by *A. tumefaciens* (Perl et al., 1996).

A novel observation in my studies was that the levels of H_2O_2 production, cell death and browning were reduced when both embryos and roots were cultured at 2.1 mM O_2 rather than at atmospheric concentration (7.4 mM). This finding is especially valuable because low O_2 tension maximizes growth of embryonic tissues (Carman, 1995; Chapter 3). I extended these findings in Chapter 4 to show that lower O_2 tension is associated with higher transformation rates by *A. tumefaciens*. I speculate that transformation frequencies

of wheat via *Agrobacterium* could be reduced by necrosis caused by bacteria-wheat tissues interaction.

Not all cells exposed to bacteria tissue become necrotic. One possible explanation for the failure of these non-necrotic cells to become transformed is that the generation of the necrotic cells activates systemic resistance in adjacent and even more distant cells and that these cells cannot undergo transformation. Necrotic cells have been observed to trigger systemic acquired resistance effective against bacterial pathogens in other systems (Glazebrook, 2001).

My findings extend the studies performed by other groups with *A. tumefaciens* of induced plant cell death (Enríquez-Obergón, et al., 1997, 1999; Perl et al., 1996; Hansen, 2000) to *A. rhizogenes*. *A. rhizogenes* can be effectively used to produce hairy roots from many tissue types, and the rapid cell proliferation and rapid growth of roots has been shown (João and Brown, 1994). For many monocot plants that cannot be regenerated *in vitro* via normal culture methods, *A. rhizogenes* can provide not only a transformation pathway for the insertion of foreign genes, but also a rapid method of plant regeneration using the built in mechanisms for hairy root induction (Tepfer, 1984). *A. rhizogenes* Ri T-DNA differs from that of *A. tumefaciens* Ti T-DNA in that the Ri T-DNA induces adventitious root formation with little or no undifferentiated cell formation as does the Ti T-DNA. Transformed roots are easily regenerated into whole plants, and they carry with them the inserted Ri T-DNA (Tepfer, 1984). Because I have observed *A. rhizogenes* interactions with wheat are similar to those *A. tumefaciens* with respect to cell death and plant root colonization, *A. rhizogenes* may also prove to be a viable vector for the transformation of monocots.

Chapter 3

1) A new plant tissue culture medium was tested and refined to increase regeneration of wheat via somatic embryogenesis;

2) reduced O_2 tension increases percentage of embryogenic callus and somatic embryos and decreases precocious germination in wheat explants.

Wheat cell culture and regeneration is one of the limiting steps to increased transformation efficiency. Due to the necrosis and cell death caused by *Agrobacterium* interactions with wheat as previously mentioned, a new set of culture parameters was delineated for wheat cell culture. In the past, wheat tissue culture was carried out on an MS based medium, and was grown at ambient O_2 levels. A new culture medium, TEC (Chapter 3), was developed which more closely resembled the *in ovulo* conditions to which immature embryo are exposed in the caryopses.

Immature wheat embryos are the optimal tissue source for regeneration of plants in tissue culture. Previous research determined that the O_2 concentrations within the caryopsis are 2.1 mM O_2, (Hess and Carman, 1993). I confirmed the hypothesis that simulation of wheat embryo growth conditions would improve somatic embryo growth and regeneration. I showed that significantly more embryogenic callus and somatic embryos were produced and fewer embryos precociously germinated in this medium and at 2.1 mM O_2 (Figure 3-1, Chapter 3). Explant responses on either TEC or MS media are improved when O_2 concentrations are reduced from 7.4 to 2.1 mM O_2. Immature embryos grown on TEC medium at 2.1 mM O_2 produce significantly more somatic embryos and fewer germinate compared to incubation at 7.4 mM O_2 (Figures 3-1, 3-2,

Chapter 3). The same is true for embryos grown on MS medium. However, on TEC medium there was a 2-fold increase in percentage of explants with embryogenic callus and somatic embryos and 83% decrease in precocious germination of the immature embryo explants.

I speculate that as an added benefit to the increased growth of somatic embryos at lowered O_2 levels, the reduction in O_2 could reduce the reactive O_2 species involved in the defense responses induced in wheat cells exposed to *Agrobacterium*. Modifications made to the traditional wheat regeneration systems outlined in Chapter 3 and the reduction of O_2 tensions and the use of antioxidants during the plant-bacterial interaction should in combination improve *Agrobacterium* transformation of cereals.

Chapter 4

1) transformation efficiencies are increased by inclusion of anti oxidant compounds and reduced O_2 levels;

2) transformation efficiencies are increased in the specialized TEC medium compared with MS medium.

The basis of this research to this point was to determine whether factors limiting plant defense mechanisms improve transformation. I suggest that wheat plants successfully defend themselves against *Agrobacterium* infection, and are thus not transformed. The plant's oxidative burst and HR-like mechanism generated in response to *Agrobacterium* would destroy cells that were in the initial stages of transformation. The remaining cells, while not directly exposed to the bacteria, are either destroyed by the

spread of H_2O_2, or are sensitized by systemic acquired resistance and somehow defended against T-DNA transfer.

Taking the information obtained in Chapters 2 and 3, I used the results as the basis for the final chapter, where I increased efficiencies in *Agrobacterium*-mediated wheat transformation. I hypothesized that the combination of improved regeneration medium and anti- O_2 treatments would improve the chances of obtaining viable, healthy transgenic wheat plants using the *Agrobacterium* system. I elected to use catalase, ascorbic acid and cysteine throughout all steps in the transformation process, from exposing the *Agrobacterium* strains to the plant material and to washing the bacteria from the explants, not just as additives in the tissue culture medium. Testing these compounds with normal and low O_2 tensions showed that the combination of anti oxidant treatments and 2.1 mM O_2 concentration increased transformation efficiencies in wheat to 6.0%, above previously published levels (Table 4-1, Figures 4-1, 4-4, Chapter 4). Overall, this research can be summarized in Figure 5-1.

Future study directions

While transformation efficiencies for wheat and other monocots continue to be low and to lag behind dicots, there is the possibility that the affects of plant defense mechanisms can be minimized using anti oxidant compounds and lowered O_2 tensions to reduce plant cell death, and to counteract the negative effects of *Agrobacterium* exposure. With a very desirable plant tissue culture medium designed specifically for wheat, and culture conditions that increase regeneration and decrease plant cell necrosis, we are ready to further investigate the *Agrobacterium*-wheat transformation system. While

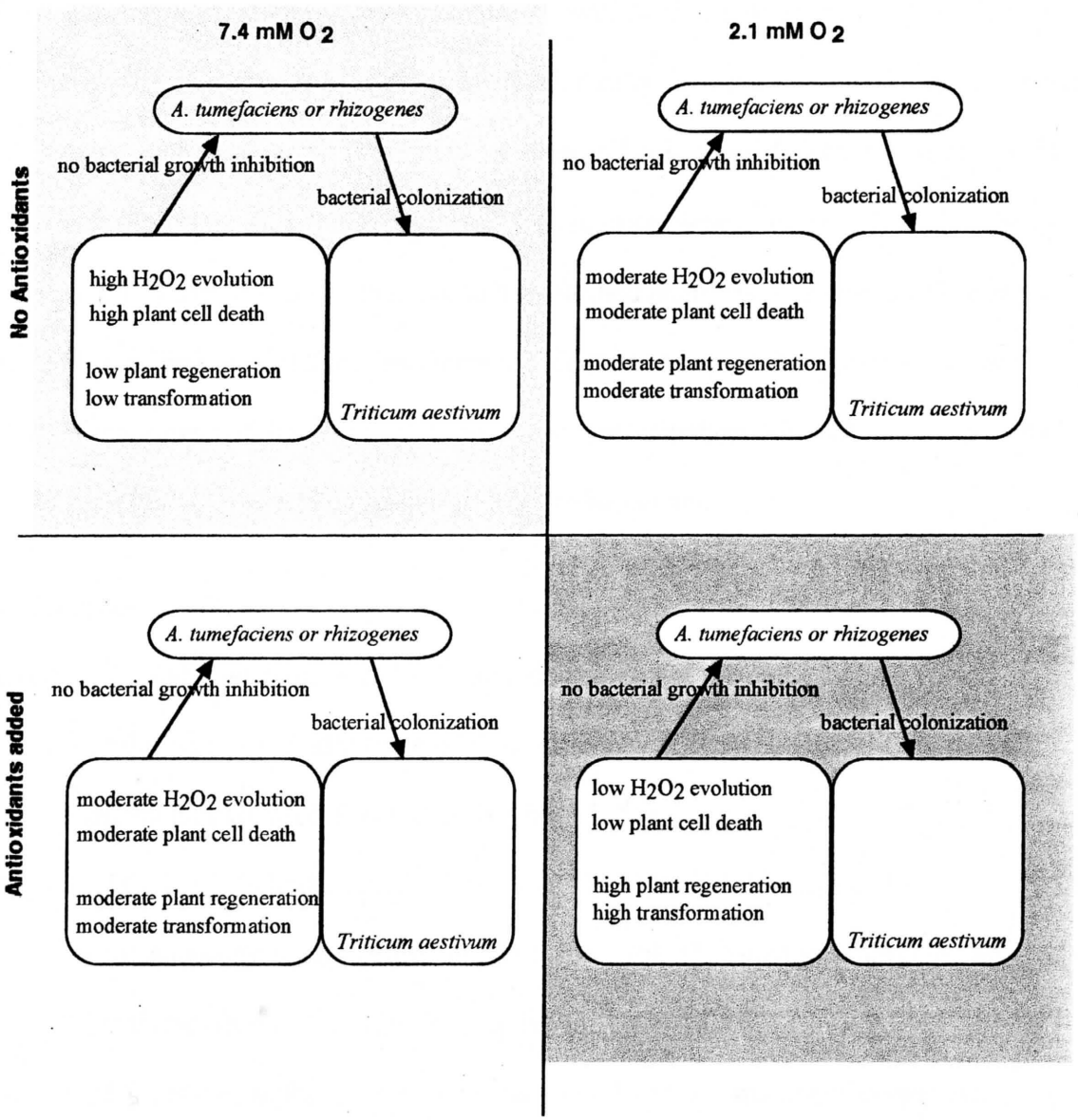

Figure 5-1. Research summary.

increased transformation in wheat has been shown, there remain many areas for further research. Are there strains of *Agrobacterium* that cause less plant cell necrosis? Can other promoters, such as monocot promoters for actin and ubiquione (Smith and Hood, 1995) increase gene expression in the plant cell? Can reporter genes, such as green fluorescent protein (GFP) increase gene detection in transformed plant cells? Additional research is needed regarding plant defense mechanism induction in monocots by *Agrobacterium.* Further measures will be necessary to increase transformation efficiency by reduction of these as of yet unknown plant responses to *Agrobacterium.*

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APPENDICES

APPENDIX A:

Agrobacterium growth in the presence of tobacco tissues

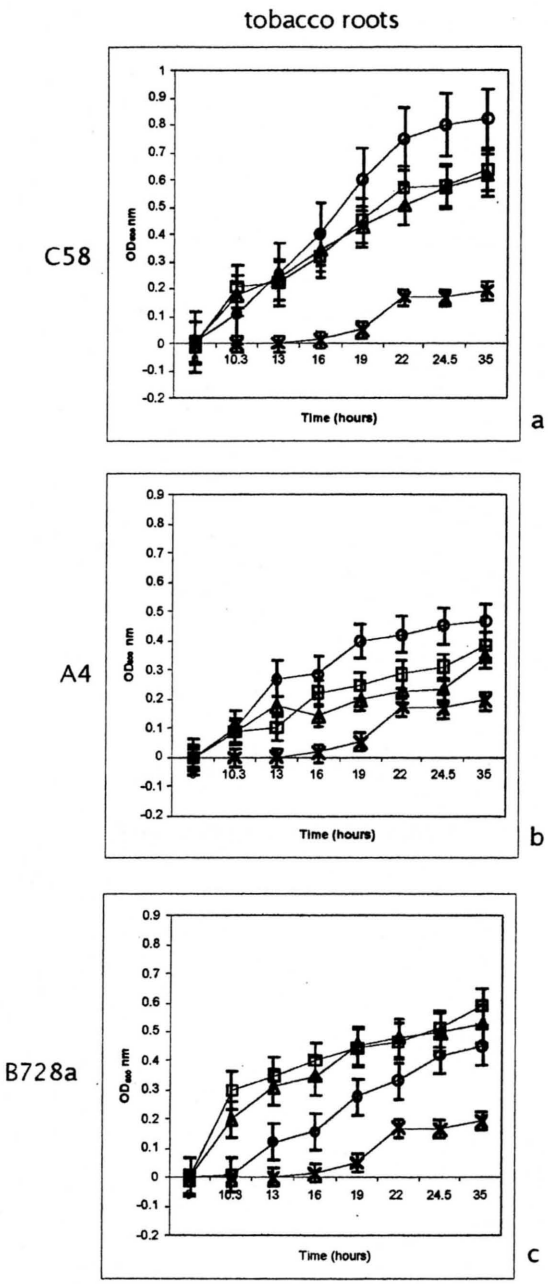

Figure A-1. Effect of tobacco factors on *Agrobacterium* growth in culture. *Agrobacterium* strains were grown in low phosphate AB glucose medium. Whole, wounded, or homogenized tobacco root tissues were exposed to bacteria. Controls contained no plant material. Tobacco roots were exposed to *A. tumefaciens* C58 (a), *A. rhizogenes* A4 (b), and *P. syringae* B728a (c). Graphs are from one study representative of six separate experiments. Error bars equal standard error from the mean for the six replicates of this one experiment. O bacteria and wounded plant tissues, □ bacteria and whole tissues, Δ bacteria and homogenized plant tissues, X, control, no plant tissues.

APPENDIX B:

Analysis of variance tables

Table B-1. Three-way ANOVA. Dependent Variable: regenerated plantlets.

Source of Variation	DF	SS	MS	F	P
O_2	1	2730.667	2730.667	288.705	<0.001
Bacteria	1	5.042	5.042	0.533	0.468
Medium	5	4750.000	950.000	100.441	<0.001
O_2 x Bacteria	1	0.0417	0.0417	0.00441	0.947
O_2 x Medium	5	523.583	104.717	11.071	<0.001
Bacteria x Medium	5	23.208	4.642	0.491	0.782
O_2 x Bacteria x Medium	5	4.458	0.892	0.0943	0.993
Residual	72	681.000	9.458		
Total	95	8718.000	91.768		

Table B-2. Three-way ANOVA. Dependent Variable: somatic embryos.

Source of Variation	DF	SS	MS	F	P
O_2	1	191441.344	191441.344	1110.811	<0.00
Bacteria	1	834.260	834.260	4.841	0.03
Medium	5	507188.052	101437.610	588.577	<0.00
O_2 x Bacteria	1	8.760	8.760	0.0508	0.82
O_2 x Medium	5	47920.719	9584.144	55.611	<0.00
Bacteria x Medium	5	3194.802	638.960	3.707	0.00
O_2 x Bacteria x Medium	5	1185.302	237.060	1.376	0.24
Residual	72	12408.750	172.344		
Total	95	764181.990	8044.021		

Table B-3. Three-way ANOVA. Dependent Variable: transformed plants.

Source of Variation	DF	SS	MS	F	P
O_2	1	3.760	3.760	16.662	<0.001
Bacteria	1	0.510	0.510	2.262	0.137
Medium	5	6.677	1.335	5.917	<0.001
O_2 x Bacteria	1	0.260	0.260	1.154	0.286
O_2 x Medium	5	3.177	0.635	2.815	0.022
Bacteria x Medium	5	4.927	0.985	4.366	0.002
O_2 x Bacteria x Medium	5	2.677	0.535	2.372	0.047
Residual	72	16.250	0.226		
Total	95	38.240	0.403		

APPENDIX C:

Permission letter

26 March 2003 Our ref: HW/ct/mar 03.J312

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Dear Mr Parrott

PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY, Vol 60, 2002, pp 59-69, Parrott et al, "Agrobacterium Induces ..."

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Research Experience

Plant-microbe interactions	*Agrobacterium*-mediated plant transformation, fluorescence and light microscopy, luminol-dependant chemiluminescent detection of hydrogen peroxide, plant cell death, cell wall biology
Plant Tissue Culture	extensive experience with monocot and dicot tissue culture, suspension culture, somatic embryogenesis, woody plant culture, regeneration of transformed cells, specialized culture medium formulation, micropropagation
DNA	plant genomic DNA isolation, PCR, miniprep, molecular cloning, sequencing, southern blotting, probe/primer preparation and troubleshooting, microsatellite DNA analysis (SSR fingerprinting) AFLP and RFLP analysis
Protein	SDS PAGE, western blotting, bench top scale protein purification using ion exchange, gel filtration, and low pressure chromatography

Professional Experience

Research Assistant, Utah State University 1994 to present
Molecular Biologist, Apomyx, Inc. January 2001 to August 2002
Teaching Assistant, Utah State University 1995-2000
Plant Micropropagation, Crop Biotechnology, Methods in Molecular Cloning, Methods in Protein Purification
Teaching Assistant, Humboldt State University 1990-1993
General Biology, General Botany, Plant Physiology, Plant Tissue Culture

Honors and Membership in Professional Societies

Member, Phi Kappa Phi National Honor Society
Presidential Scholar, Utah State University 1997
Outstanding Student Award, Humboldt State University 1989, 1990, 1991
Member, American Society of Plant Biologists

Abstracts and Papers Presented at Professional Meetings

Parrott DL, Anderson AJ, Carman JG. 1997.Physiological and Biochemical Aspects of *Agrobacterium*-Wheat Interactions. p. 27 In Plant Biology '97 Final Program, supplement to PlantPhysiol 113:3

Parrott DL, Anderson AJ, Carman JG. 1998. *Agrobacterium* interactions with wheat (*Triticum aestivum* L.): Bacterial growth, resistance, and plant cell death. Poster presented at the 1998 ASA annual meetings, Baltimore, MA.

Publications

Parrott DL. 1993. The Micropropagation of Sycamore, *Acer pseudoplatanus*, by use of organogenesis and shoot-tip culture techniques. Master's Thesis, Humboldt State University, Arcata, CA.

Parrott DL, Anderson AJ, Carman JG. 2002. *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.). *Physiological and Molecular Plant Pathology.* **60**:59-69

Parrott DL, Carman J G.2003. An improved medium for *in vitro* wheat (*Triticum aestivum* L.) embryo culture. In preparation

References

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