Characterizing the factors contributing to intraspecific pollen-mediated gene flow between cropped and volunteer spring wheat (*Triticum aestivum* L.)

by

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

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

Transgenic crops have been widely adopted throughout many parts of the world. Their popularity is not without concern regarding the ecological and agricultural quandaries associated with their cultivation. Several traits have recently been incorporated into hexaploid wheat (*Triticum aestivum* L.) using recombinant DNA technology and therefore, the resulting transgenic lines may require segregation from non-transgenic wheats to satisfy international markets. However, segregation may be complicated by the underlying biological characteristics of wheat plants that collectively allow for the transfer of genes between cropped and volunteer spring wheat populations. To this end, the objectives of this thesis were: i) to identify a hybridization window in spring wheat crops that would facilitate temporal isolation, thus minimizing flowering synchrony and pollen-mediated gene flow (PMGF); ii) to determine whether increasing crop density of spring wheat crops which are taller than volunteer genotypes could reduce flowering synchrony and PMGF between the two populations and; iii) to ascertain whether a relationship exists between flowering synchrony and PMGF such that flowering synchrony could be used to predict PMGF. Synchrony of flowering and PMGF were always greatest when seedling emergence timing of volunteers coincided with the wheat crop. Flowering synchrony ranged between 0% and 86% depending on emergence timing, and a distinct hybridization window 125 GDD in length was indentified in which seedling emergence of volunteer wheat plants would result in PMGF regardless of volunteer density. Volunteers emerging after the wheat crop (which cannot be selectively controlled) contributed substantially to PMGF and although plant height appeared to have some merit in reducing flowering synchrony, it had no influence on PMGF. Increasing crop density, on the other hand, resulted in an exponential decline in PMGF in all site-years. However, further reductions above the recommended planting rate of 250 plants m^{-2} were not realized. Overall, this research suggests that the potential for PMGF between volunteer wheat plants and a wheat crop is real and perhaps absolute. Nevertheless, results presented in this thesis lend support for the possibility of coexistence between transgenic and non-transgenic wheat within an appropriate framework that includes the establishment of realistic thresholds for admixture.

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This work is dedicated to my grandparents, Bernice and August Willenborg and James and Julia Swab, whose passion for, and dedication to agriculture, serve as a vivid reminder that "Cultivators of the earth are the most valuable citizens. They are the most vigorous, the most independent, the most virtuous, and they are tied to their country and wedded to its liberty and interests by the most lasting bands" (Thomas Jefferson, 1785).

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List of Abbreviations

AC	Agriculture and Agri-Food Canada
ANOVA	Analysis of variance
ALS	Acetolactate synthase
AP	Adventitious presence
BC	Backcross
CAR	Carman (Ian Morrison) Research Farm
CDC	Crop Development Center
CDF	Cumulative density function
CPS	Canada prairie spring
CWES	Canada western extra strong
CWRS	Canada western red spring
D	Density
DAT	Days after treatment
DNA	Deoxyribonucleic acid
ET	Emergence time
G	Genotype
GDD	Growing degree days
HR	Herbicide-resistant
IMI	Imidazolinone
IR	Imidazolinone-resistant
PDF	Probability density function
PMGF	Pollen-mediated gene flow

R	Resistant
S	Susceptible
TKW	Thousand kernel weight
WPG	Winnipeg (JRI Kelburn) Research Site

1.0 Introduction

Wheat (*Triticum aestivum* L.) was one of the earliest domesticated crops grown for human consumption, having originated in the 'Fertile Crescent' region of southwest Asia during the dawn of agriculture 10,000 to 12,000 years ago (Kimber and Sears, 1987). Widespread cultivation of wheat originally occurred upon recognition of the dough-like properties of its grain (Poehlman and Sleper, 1995). Wheat is also planted to a limited extent as a forage crop for livestock, and the straw also can be used as fodder for livestock. The cultivation of wheat traditionally has been of significant importance in Canadian agriculture, and the acreage devoted to wheat has remained fairly constant over the past three decades, with ca. 38% of arable land in Canada sown to wheat (Statistics Canada, 2006).

Conventional wheat breeding has posed many challenges to wheat breeders (Poehlman and Sleper, 1995). To overcome these limitations, desirable genes have been isolated from other organisms and inserted into specific wheat varieties via genetic engineering. Genetic engineering has provided the opportunity to potentially insert an unlimited number of novel traits into plants to exploit various opportunities and overcome previously impossible challenges (Tolstrup et al. 2003). On a global scale, the production of transgenic crops is large and growing, increasing from 67.7 million hectares in 2003 to 114 million hectares in 2007 (James, 2007). Bread wheat is the most prevalent crop grown for and consumed by humans in which transgenic cultivars are currently under development (Braun et al., 1998). Different types of transgenic wheat may be ready for commercialization within the next decade including varieties with higher yields, greater resistance to biotic and abiotic stresses, and resistance to herbicides. Several groups have,

however, expressed concern about the large scale cultivation of transgenic crops. One of the most important risks associated with transgenic crops is transgene movement, which can result in genetic admixture and the adventitious presence of transgenic crop seeds in material destined for non-transgenic markets (Van Acker et al., 2003). Therefore, it is important to understand the movement of a transgenic trait within the agronomic production system before transgenic wheat is released (Brûlé-Babel et al., 2006). Nevertheless, transgene movement remains inadequately understood (Marvier and Van Acker, 2005), particularly for intraspecific movement of transgenes within and among farming systems (Tolstrup et al. 2003).

Adventitious presence (AP) refers to "the unintentional presence and accidental commingling of trace amounts of "off-types" of seed or grain in a parcel of seed or grain" (Demeke et al., 2006). Adventitious presence primarily occurs as a result of cross pollination and seed commingling during harvest, transport, or storage of grains. The continued appearance of off-types in wheat cultivars indicates that genetic purity can be compromised (Hucl et al., 2004) despite the primarily self-pollinating nature of wheat (Waines and Hedge, 2003). Similarly, concerns over market acceptance of transgenic wheat and the importance of protecting patented plant cultivars necessitates heightened attention around the issue of crop to crop and crop to volunteer gene flow (Conner et al., 2003; Kershen, 2004). The relatively high frequency of wheat in North American crop rotations is concerning with respect to transgene movement and the potential for contamination (Leeson et al., 2002a,b, 2003; Van Acker et al., 2003) because it is likely that many transgenic wheat cultivars will be grown next to non-transgenic wheat cultivars. Moreover, volunteer wheat plants also may serve to facilitate transgene movement. Therefore, a thorough understanding of pollen-mediated gene flow and the factors that contribute to it are essential to ensure cultivar purity and keep adventitious presence below threshold levels.

The factors that contribute to intraspecific transgene movement in spring wheat in western Canada have been identified (Van Acker et al. 2003). Mechanistic and deterministic models of transgene movement at field scales have already been created for winter oilseed rape in Europe (GeneSys; Colbach et al. 2001a, b). In western Canada, the

large number of wheat acres present will likely increase seed comingling, but it is the mechanisms behind the movement of transgenes and the resulting genetic admixture which must be examined if we are to minimize gene flow. It is therefore critical to determine the potential for transgene movement and resulting genetic contamination utilizing a proactive modeling approach. However, before these models can be developed, a thorough understanding of the biological factors contributing to intraspecific gene flow in wheat must be developed. Currently, critical information is lacking with regard to flowering synchrony between wheat crops and volunteers, and consequently, little is known about gene flow between the two.

The focus of this project was to assess the influence of various agronomic factors on the phenological characteristics that contribute to intraspecific pollen-mediated gene flow between cropped and volunteer spring wheat populations. More specifically, the research aimed to determine whether asynchronous emergence of volunteer populations at various densities also results in asynchronous flowering and reduced overlap, thereby resulting in lower frequencies of pollen-mediated gene flow. In addition, the research was to assess the merit of growing tall genotypes at high densities to reduce flowering synchrony and gene flow between volunteer and cropped spring wheat. The primary hypothesis was that flowering synchrony and hence, gene flow, would be highest where semi-dwarf genotypes were grown at low crop densities and volunteer wheat emergence (at high densities) coincided with that of the crop. The following key objectives were addressed:

(1) Determine the sensitivity of several spring wheat genotypes of different parental backgrounds and market classes to imazamox at the whole-plant level
(2) Determine the influence of volunteer wheat density and emergence timing relative to cropped wheat on flowering phenology and synchrony
(3) Examine the potential for temporal isolation, as a function of volunteer emergence time relative to the crop, to influence pollen-mediated gene flow (PMGF) in spring wheat and assess how density affects this relationship
(4) Examine the influence of crop density, genotype, and height on flowering phenology and synchrony between volunteer and cropped spring wheat populations

(5) Determine the effect of crop height, genotype, and crop density on pollen-mediated gene flow between volunteer and cropped spring wheat
(6) Determine the nature and strength of the relationship, if any, between PMGF, flowering synchrony, and yield components in spring wheat

Answers to these questions will provide information to develop recommendations for producers, agronomists, and breeders to minimize pollen-mediated gene flow between cropped and volunteer wheat. Question one will provide researchers with an estimation of the value of using imazamox-resistant wheat as a phenotypic marker in gene flow studies. Questions two and three will quantify flowering synchrony and PMGF over a range of volunteer emergence times and densities. The potential of taller genotypes and increased crop densities to minimize flowering synchrony and the resulting PMGF is addressed in questions four and five. Question six brings questions two through five together by assessing the nature of the relationship between flowering synchrony and gene flow and should determine whether flowering synchrony is a good predictor of PMGF in wheat. Taken together, these studies will provide information regarding the potential movement of transgenes between cropped and volunteer wheat and should provide the first estimates of the contribution of volunteers to PMGF in any crop. These are also the first studies to quantify flowering synchrony between crops and volunteers and to link this data to gene flow. The information gathered in this thesis will be valuable to producers, agronomists, and regulators for the development of coexistence plans. In addition, data from this project will serve to parameterize and facilitate the development of coexistence models based on population dynamics and gene flow.

2.0 Literature Review

2.1 Taxonomy and genetics of hexaploid wheat

Hexaploid wheat is believed to have originated in the 'Fertile Crescent' of SW Asia (Vavilov, 1951). Hexaploid wheat (*Triticum aestivum*) is an allohexaploid with $2n = 6x = 42$ chromosomes that generally forms 21 pairs of chromosomes during meiosis (Kimber and Sears, 1987). It has three homoeologous genomes, A, B, and D, each composed of seven pairs of homologous chromosomes (AABBDD). The genome of hexaploid wheat is large, estimated at 16 billion base pairs per haploid nucleus (Bennett, 1972). Most of this (75%) contains repeated DNA sequences varying both in length and the amount of repetition, while about 25% is comprised of unique sequences (Smith and Flavell, 1975). Actual coding regions comprise only about 1% of the genome (Kimber and Sears, 1987).

Hexaploid wheat is believed to have originated from the wild form of einkorn (*Triticum monococcum* L. sensu lato, AA) (Feldman, 1976) through two hybridization and spontaneous chromosome doubling events. First, *T. monococcum* combined with the donor of the B genome (*A. speltoides* (Taush)) to form a tetraploid ($2n = 4x = 28$, AABB) (Kimber and Sears, 1987; Anonymous, 2005a; Petersen et al., 2006), followed by hybridization of the tetraploid and *Aegilops tauschii* (Coss.) Schmal., the progenitor of the D genome. Most of the wild and cultivated wheats are one of three species, *T. compactum* Host. (club wheats), *T. turgidum* L. ssp. *durum* (Desf.) Husn. (durum wheats), or *T. aestivum* (hexaploid wheat), the latter being the most commonly cultivated species worldwide.

2.2 Wheat cultivation in Canada

Hexaploid wheat is an important crop for domestic consumption and export in many countries throughout the world, including Canada. Grown on approximately 38% of the arable land utilized for crop production, hexaploid wheat is Canada's most widely grown crop (Statistics Canada, 2006). In 2005, there were approximately 25.6 MMt of wheat produced on 9.8 Mha in Canada (FAOSTAT, 2006) (Table 2.1). In comparison, 97 MMt were harvested on 22 Mha in China and 57.1 MMt were harvested from 20 Mha in the United States of America (USA). Canadian yields in 2006 averaged 2.6 t ha^{-1}, slightly below the world average of 2.9 t ha^{-1}. This is attributable to the semi-arid climate and dryland production of the majority of the wheat crop in Canada.

According to Statistics Canada, greater than 97% of the wheat produced in Canada is grown in the Prairie provinces, with Saskatchewan, Alberta, and Manitoba producing 56%, 26% and 15% of Canada's wheat, respectively (Statistics Canada, 2001). Spring wheat represented 76% of the wheat acreage in Canada between 1981 and 2001, durum wheat accounted for 20%, and winter wheat constituted 3% (Statistics Canada, 2001). Hard red spring wheat is the most widely grown market class of wheat, comprising 65% of the wheat crop in the Prairie provinces (Canadian Wheat Board, 2008). Although the area of wheat harvested has declined steadily from 14.1 Mha in 1990 to 9.8 Mha in 2005 (FAOSTAT, 2006), wheat remains the most widely grown crop in western Canada (Leeson et al., 2005). Therefore, the introduction of transgenic wheat could have a significant impact on a large portion of the cultivated land in Canada, both with respect to gene flow and especially seed admixture.

Crop-to-crop and volunteer-to-crop hybridization would be difficult to contain on such a large scale if stringent thresholds are to be upheld; the chance of seed comingling among the nearly 26 MMt of wheat produced in Canada is high and perhaps inevitable. For example, in the USA where average annual rice (*Oryza sativa* L.) crop production is ca. 9 MMt per year (FAOSTAT, 2006), already one case of widespread AP of transgenic rice has occurred. Liberty Link 601, a herbicide-resistant (HR) genotype of rice, was grown in test plots between 1998 and 2001 but was discovered in storage in

TABLE 2.1 Production, area harvested, and yield of the 10 highest wheat producing countries in the world in 2005†

Country	Production (Mha)	Area Harvested (Mha)	Yield (t ha^{-1})
Australia	24.1	11.4	2.2
Canada	25.5	9.8	2.6
China	96.3	22.5	4.3
France	36.9	5.3	7.0
Germany	23.6	3.2	7.4
India	72.0	26.3	2.7
Pakistan	21.6	8.3	2.6
Russian Federation	47.6	23.0	2.1
Turkey	21.0	9.3	2.3
USA	57.1	20.2	2.8
World	**628.1**	**215.6**	**2.9**

†Source: FAO, 2006.

Missouri, Arkansas, and as far away as Europe in 2006 (Vogel, 2006). Similarly, Canadian production of canola and rapeseed (*Brassica napus* L.) has been ca. 7.5 MMt annually (FAOSTAT, 2006) since 1996 and in 10 yrs has experienced two known cases of AP involving transgenic seeds in non-transgenic seedlots (Demeke et al., 2006). Gaines et al. (2007b) report that AP of non-transgenic imazamox-resistant winter wheat seed ranged from 0 – 11.28% in Colorado, with one certified seed sample and three farm-saved samples exceeding the 0.1% threshold for off-types in certified wheat seed. The previous examples highlight the difficulties in ensuring coexistence, and a continued rise in AP can be expected with the continued introduction of transgenic crops.

2.3 Seedling biology and weediness of *T. aestivum*

Triticum aestivum is an annual plant that is grown primarily in temperate climates. Most of the wheat planted in Canada is of the spring type, with only 3.8% of seeded acres being the winter type (Statistics Canada, 2001). Because these crops are planted primarily for grain production, the harvesting operation can disperse wheat seeds into favorable microsites resulting in recruitment during intervals between crops, creating volunteer wheat plants. Volunteers are defined as "those plants that occur (in a population) not as a result of current planting of the crop under consideration, but from seeds growing uncontrolled from previous planting or from plants escaping cultivation and scattered by natural means" (Barnes and Beard, 1992). These volunteers can become problematic due to their potential to reduce yield and the difficulty associated with controlling them in crop production systems (Gressel, 2005). Additionally, volunteer wheat can serve as a green bridge or source for the transmission of wheat diseases and insects from previous to future wheat crops (Lyon et al., 2002). More recently, concern has centered on the potential of volunteer wheat to facilitate intraspecific (within species) and interspecific (between species) trait movement to non-transgenic (conventional) wheat crops. Commercial cultivation of transgenic wheat in Canada would require strict control of volunteers if thresholds for trait confinement are to be maintained (Marvier and Van Acker, 2005; Brûlé-Babel et al., 2006).

In concert with the large acreage seeded to wheat in western Canada, surveys of western Canada indicate that the relative abundance of volunteer wheat (averaged across Alberta, Manitoba, and Saskatchewan) has increased from a rank of 31 (out of 164) in the 1980s to 22 (out of 149) in the 1990s to 12 (out of 148) in the early 2000s (Leeson et al., 2005). During that time, the frequency of fields in which volunteer wheat was observed after in-crop herbicide applications increased from 5.1% in the 1980s to 9.3% in the 1990s to a present day frequency of 10.8% at an average density of 6 plants m^{-2}. Moreover, the relative frequency, relative uniformity, and relative density of volunteer wheat have increased substantially for spring wheat in western Canada over the past three decades (Leeson et al., 2005). This is likely attributable to an increase in continuous cropping and decrease in fallow (Campbell et al., 2002), as volunteer wheat is known to be associated with continuous cropping (Derksen et al., 1994). Little is known regarding the association of volunteer wheat with specific cropping systems and management practices such as tillage intensity and time of weed removal and further investigation in this area is warranted. Moreover, surveys cannot distinguish wheat volunteers in wheat crops (the most frequently planted crop in western Canada) and therefore, the above survey values likely underestimate the true level of volunteer wheat infestation.

2.3.1 Seedbank Additions

The routes for wheat seeds to enter the seedbank are through planting, seed contamination, and via seed losses during wheat harvest. The latter may occur in the form of seed rain (shattering or natural dehiscence of the seed from the spike), or through mechanical seed losses incurred during harvesting. An extensive study of harvest losses in spring wheat in western Canada conducted by Clarke (1985) indicated that total harvest losses, including natural shattering and mechanical harvest losses, varied widely from approximately 35 to more than 800 seeds m^{-2} (almost four times the normal seeding rate). Inconsistent variation precluded Clarke (1985) from providing a numerical average for overall wheat harvest losses but it is anticipated to be in the order of 300 seeds m^{-2} (De Corby et al., 2007). This value is similar to UK data which estimates wheat harvest

losses at 2% or approximately 240 plants m^{-2} (Anderson and Soper, 2003). Studies conducted by the Prairie Agricultural Machinery Institute in SK estimate harvest losses in Canada average between 3 to 5% (Les Hill, PAMI, personal communication). Natural shattering can contribute to large harvest losses as well, particularly if harvest is delayed as shattering increases with plant maturity. Pickett (1993a) observed natural shattering losses as high as 16%, but results varied substantially among cultivars. Grain loss during harvest is also reported to vary with the type and speed of combine harvester (Komatsuzaki and Endo, 1996).

Seedbank additions also occur as a result of seed losses from external factors such as hail damage and wheat stem sawfly (*Cephus cinctus* Norton), which causes plants to lodge. Losses due to hail damage are difficult to quantify, but have been implicated in the persistence of volunteer wheat populations (Harker et al., 2005). *Cephus cinctus*, on the other hand, has historically been a major pest of spring wheat in Canada. Beres et al. (2007) reported wheat losses due to lodging caused by sawfly among western Canadian cultivars as high as 20% or 460 seeds m^{-2}. Similarly, losses in Anatolia caused by sawfly (*C. pygmaeus* L.) infestation were found to be 2.2% (approximately 600 seeds m^{-2}) in durum wheat and 3.3% (approximately 900 seeds m^{-2}) in hexaploid wheat (Özberk et al., 2005). While solid-stemmed varieties exhibit significantly fewer seed losses than hollow-stemmed varieties, these losses still exceeded 5% (Beres et al., 2007), suggesting that harvesting losses may be only half that caused by sawfly infestations. Furthermore, the majority of wheat cultivars currently grown in Canada are susceptible to sawfly damage (Beres et al., 2007). Seedbank additions may also occur from light weight kernels such as those damaged by orange-blossom wheat midge (*Sitodiplosis mosellana* Gehin) or fusarium head blight (*Fusarium graminearium* Schwabe), which are distributed back onto the soil surface by the harvester. However, these seeds are generally of low quality, exhibiting both reduced germination capacity and viability (Lamb et al., 2000; Argyris et al., 2003). Consequently, they are less likely to contribute to volunteer recruitment, although their contribution to the persistence of volunteer populations remains unknown.

Wheat seed that enters the field through contamination or that is not removed from the field at the time of harvest becomes part of the seedbank (Fig. 2.1). In the seedbank, volunteer wheat seeds are subject to several fates including immediate germination, quiescence or a short dormant period followed by germination, predation, microbial decomposition, or seed death (Anderson and Soper, 2003). Wheat seeds that enter a period of quiescence and/or dormancy, followed by recruitment at a later time are of greatest concern because these produce the volunteer population. Although no evidence exists to support a sustained level of secondary dormancy in wheat, studies have demonstrated persistence of volunteer wheat ranging from one to five years (Beckie, 2001; Harker et al., 2005; Beckie et al., 2006; De Corby et al., 2007). Given that wheat is grown so frequently and on vast acreage, even persistence of two or more years may be of concern with respect to the potential for crop-to-volunteer transgene movement.

Feral wheat populations represent another potential vector for intraspecific transgene movement. Feral populations arise when domesticated plants evolve to become untamed or undomesticated (Gressel, 2005). Often what occurs is that the life cycle of volunteer plants that have successfully established in non-crop areas is completed before the seedbank is depleted entirely (Gressel, 2005). A wild relative of wheat, feral rye (*Secale cereale* L.) populations have been identified in several non-crop areas throughout the mid-western USA (Stump and Westra, 2000; Ayal and Levy, 2005). While feral wheat populations could theoretically become established on roadsides and waste areas in the Prairie provinces (Ayal and Levy, 2005), Leeson et al. (2002b) found that volunteer wheat was a major weed in fields and at field edges, but did not occur in roadside ditches. Nevertheless, the invasion success of feral wheat populations at the field edge into fields has not been investigated. To the best of my knowledge, however, feral wheat populations have not been identified in Canada.

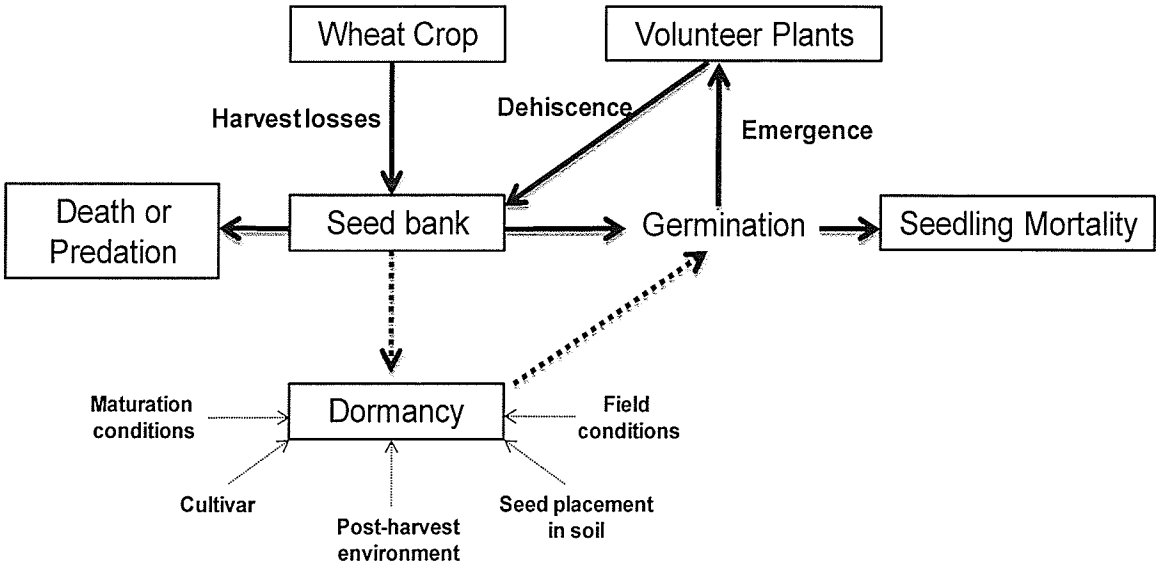

FIGURE 2.1 Schematic representation of the life cycle of volunteer wheat. Dashed arrows represent transitional states while dotted arrows represent exogenous factors contributing to persistence.

2.3.2 Germination and Establishment Characteristics

Seed germination characteristics of wheat may directly influence volunteer seedling recruitment in the field. Several studies have reported a base temperature of 0°C for wheat germination and growth, with little variation between winter and spring cultivars (Baker et al., 1980; Bauer et al., 1984; Cao and Moss, 1989a). The germination rate of wheat seed increases with increasing temperatures, but final germination percentages are greater at 10 to 15°C than 25 to 30°C (Wilson and Hottes, 1927; Reddy et al., 1985). Nyachiro et al. (2002) also reported that 10 to 15°C temperatures were more conducive to greater germination than temperatures of 25 to 30°C among 10 Canadian wheat cultivars. While temperatures between 12 to 25°C appear to be optimum, germination may occur between temperatures as low as 4°C and as high as 37°C (Austin and Jones, 1975).

As would be expected, decreased water potentials adversely affect the germination characteristics of wheat. In the laboratory, Lafond and Baker (1986) observed little difference in final percent germination among various osmotic potentials, but noted that germination was delayed significantly at lower water potentials among wheat seed of various sizes. Lafond and Fowler (1989) demonstrated that temperature had a greater effect on the speed of germination than moisture. As soil temperature increased, median germination time decreased significantly, much more so than at different water potentials. Similarly, seed germination for six wheat cultivars declined progressively in response to decreasing water potentials (Singh, 2001). In addition to reducing and delaying germination, more negative water potentials can also contribute to reduced seedling root and shoot weights (Baalbaki et al., 1999).

Several field studies have indicated that the quantity and timing of recruitment varies substantially (Anderson and Nielsen, 1996; Wicks et al., 2000; Harker et al., 2005; De Corby et al., 2007). Data from the USA has shown that volunteer winter wheat emerged periodically throughout the growing season in a number of cohorts (Anderson and Nielsen, 1996). Final seedling emergence in the study (over four years) ranged 16-fold from 28 to 423 plants m^{-2}, suggesting that volunteer wheat can become problematic if conditions are favourable for successful recruitment. Furthermore, greater than 40

volunteer winter wheat seedlings m^{-2} emerged more than 14 months after entering the seedbank (Anderson and Nielsen, 1996). Likewise, Harker et al. (2005) observed the majority of wheat seedling recruitment in the year following seed dispersal, with a small proportion of seedlings recruited up to two years following seed dispersal (Harker et al., 2005). Extraordinary reseeding events due to hail and *C. cinctus* damage were reported to lengthen persistence. Tillage also affected recruitment, with greater volunteer wheat densities observed in seeding systems with higher levels of disturbance. In contrast to these results, De Corby et al. (2007) observed little variation in the timing of recruitment and the effects of tillage and pre-harvest sprouting characteristics on volunteer spring wheat recruitment. Emergence in the De Corby et al. (2007) study generally occurred as a single, uniform cohort beginning approximately 200 growing degree days (GDD) into the growing season; total cumulative emergence ranged between 0.9 to 13.1%.

Discrepancies between the studies noted above may be due to several factors, including differences in precipitation levels and tillage regimes. Precipitation levels varied greatly between the studies and indeed, higher volunteer wheat densities were recorded at sites with lower annual precipitation in one of the studies (Harker et al., 2005). Tillage is known to affect weed and volunteer crop seed placement in the soil, microsite availability, persistence, and timing of seedling emergence (Bullied et al., 2003). Cumulative emergence of wheat seed from the soil surface was 55% compared with 85% at a depth of 3-4 cm (Boyd and Van Acker, 2003). Although the effects of tillage on volunteer wheat persistence are well-known for winter wheat (Anderson and Soper, 2003), little is known in regard to spring wheat.

Considerable variability clearly exists in volunteer wheat seed germination, emergence, and recruitment characteristics. The quantity and the timing of recruitment, as well as the survival of recruits may have substantial effects on volunteer-to-crop gene flow. Increased recruitment could lead to higher in-crop volunteer densities, thus increasing pollen source size. Therefore, the processes of germination and recruitment of volunteer wheat could profoundly affect trait movement. Ideally, the development of a hydrothermal time model to predict wheat emergence would facilitate mechanistic gene

flow modeling efforts as well as expedite the development of best management practices for trait confinement in hexaploid wheat.

2.3.3 Seedbank Persistence

Persistence may be defined as the survival of a plant or population when exposed to a variety of environmental rigors (Thompson et al., 2003). Seedbank persistence of wheat can be attributed to a number of factors including reseeding events, environmental conditions, or seed dormancy (De Corby et al., 2007). It is widely assumed that seeds require some form of dormancy if they are to persist in the soil seedbank; however, persistence does not necessitate dormancy and the two are not synonymous (Thompson et al., 2003).

Wheat seeds can possess a sustained level of primary dormancy, rendering them dormant at maturity. The incorporation of some degree of initial seed dormancy in wheat cultivars is desirable to prevent pre-harvest sprouting (Pickett, 1993a; Nyachiro et al., 2002; Matus-Cádiz and Hucl, 2003). Indeed, several wheat lines (e.g. W98616, line 211, CDC EMDR-4, and CDC EMDR-14) possessing varying levels of primary seed dormancy have been released in Canada (Hucl and Matus-Cádiz, 2002a,b,c). Wheat genotypes exhibit varying dormancy levels relative to their genetic background (Reddy et al., 1985; McCaig and DePauw, 1992; Matus-Cádiz and Hucl, 2003). Nevertheless, De Corby et al. (2007) observed little seedbank persistence of spring wheat in Manitoba when freshly-harvested kernels were broadcast in the fall and moisture conditions were adequate, regardless of genetic background.

Several factors affect the development of seed dormancy in wheat genotypes. Komatsuzaki and Endo (1996) observed a six-fold variation in the duration of dormancy among several Japanese cultivars. Endogenous factors such as seed coat inhibitors induced dormancy in a number of red-grained wheat cultivars (McCrate et al., 1982). Extreme environmental conditions such as high temperature and low moisture conditions during seed maturation and germination also increased primary dormancy in wheat

(Pickett, 1993a). Temperatures ranging between 10 and 15°C tended to favor wheat seed germination, whereas a temperature of 30°C promoted dormancy among 10 wheat genotypes (Nyachiro et al., 2002). Similarly, high nitrogen application rates and seed nitrogen contents promoted dormancy, although results were inconsistent among cultivars and environmental conditions (Morris and Paulsen, 1985).

Numerous studies have demonstrated that volunteer wheat populations can persist at low levels (Beckie, 2001; Anderson and Soper, 2003; Harker et al., 2005; De Corby et al., 2007); however, the conclusions reached by these studies vary considerably. Classic seed burial studies suggest that few wheat kernels remain viable after one year, while field studies have demonstrated seedbank persistence of up to two years following wheat harvest (Anderson and Soper, 2003). Consistent with these results, De Corby et al. (2007) noted that volunteer wheat seed of eight spring wheat cultivars rapidly degraded in the soil and did not persist for more than 1 yr. Harker et al. (2005) observed wheat persistence at low densities up to 3 yr after seed dispersal at eight sites across western Canada. Cropping systems with greater levels of disturbance generally were found to have greater wheat densities, but only at one site were preplanting densities sufficient to warrant a herbicide application in addition to glyphosate (Harker et al., 2005). While Harker et al. (2005) concluded that there was little evidence that wheat seed persistence will become problematic from an agronomic perspective, indirect evidence from weed survey data indicate that volunteer wheat can persist for as long as 5 yrs (Thomas and Leeson, 1999; Beckie, 2001). The mechanisms underlying this persistence have not been elucidated and it is unclear whether seed dormancy or reseeding by plants that escape control is the cause (Thomas and Leeson, 1999). Further to this, both the Harker et al. (2005) and De Corby et al. (2007) studies were conducted by scattering seeds on the soil surface vs. burying seeds, but the longevity of seeds in unthreshed ears is known to be greater than that of loose seeds in the soil (Komatsuzaki and Endo, 1996).

Wheat seed longevity will be influenced by both the level of primary dormancy and the quantity of shed seeds relative to those still attached to unthreshed ears. Studies examining the potential for induction of volunteer wheat seed into secondary dormancy are not found in the literature. It is conceivable that because wheat seeds are enveloped in

a soft seed coat and contain a free-threshing hull that they are susceptible to rapid decomposition in the soil seedbank and may even be more prone to predation, particularly in moist soils (Kremer, 1993). Indeed, the pygmy field mouse (*Apodemus microps* Krat. and Ros.) and the wood mouse (*A. sylvaticus* L.) prefer seeds of wheat over those of barley (Heroldová et al., 2007). However, wheat seed consumption was not quantified in the study. It is also not known whether genotypes possessing enhanced levels of sprouting resistance also possess the ability to be induced into secondary dormancy. More research is needed regarding the seedbank dynamics of volunteer wheat and should be aimed at addressing the aforementioned knowledge gaps. Finally, studies are needed to examine longevity among genotypes with enhanced levels of sprouting resistance that compare shed seeds with that of seeds in unthreshed ears.

The potential for transgene movement occurs at both temporal and spatial scales. Pollen-mediated gene flow or seed movement of wheat constitute the spatial aspect of transgene movement, while volunteer wheat populations facilitate transgene movement in time. In either case, volunteer populations could play a crucial role in trait movement. Volunteer wheat commonly survives to flowering at significant occurrence densities in a significant proportion of fields in western Canada (Leeson et al., 2002a,b), a very important observation that deserves more attention. Volunteer wheat can also persist, emerge, and flower in subsequent wheat crops (Beckie, 2001). Therefore, understanding the extent and nature of volunteer wheat is critical to understanding the potential mechanisms of transgene movement in wheat.

Another potential concern regarding transgenic wheat is the control of herbicide-resistant volunteers. The problem arises because broad-spectrum herbicides such as glyphosate could no longer be used to control glyphosate-resistant volunteers. While tillage can be used as an effective control measure, this is not an option for zero-tillage producers (Van Acker et al., 2003). Wheat is susceptible to a number of herbicides but volunteers can compete strongly with the following crops if not adequately controlled (Rainboldt et al., 2004; Blackshaw et al., 2006; De Corby et al., 2007). Paraquat, clethodim, and quizalofop-P provided adequate control of glyphosate-resistant volunteers (Rainboldt et al., 2004; Blackshaw et al., 2006). Substitution of paraquat for glyphosate

in a preseed burnoff, however, would increase costs from approximately $11 to $48 per hectare (Anonymous, 2007). Similarly, clethodim and quizalofop-P must be tank-mixed with glyphosate for a preseed burnoff, and would add approximately $27 and $30 ha^{-1}, respectively (Anonymous, 2007). These represent significant increases in cost and therefore, further studies are needed to develop alternative methods of volunteer HR wheat management.

2.4 Reproductive biology of *T. aestivum*

2.4.1 Inflorescence Initiation and Variation

The majority of wheat flowers are hermaphroditic; however, some unisexual female flowers are often present near the basal and terminal ends of the spike and represent potential sites for gene flow to occur (De Vries, 1971). Each floret contains three anthers and two feathery stigmas enclosed within the glumes consisting of the palea and lemma. Glume opening is controlled mainly by turgor pressure and regulated largely by the lodicules (Kimber and Sears, 1987).

Great variation exists between wheat cultivars with respect to spike size, shape, and density. The palea and the lemma in compact spikelets are known to open at a lower angle compared to those in lax-headed wheats (Johnson et al., 1967). Often referred to as spike laxness, the degree of separation is an important factor influencing outcrossing and gene flow in wheat (Hucl, 1996; Hucl and Matus-Cádiz, 2001). In fact, Waines and Hegde (2003) cite the extent of flower opening as the most important influence on cross-pollination and potential gene flow during anthesis. As flower opening increases, so too does the potential for the flower to become a pollen donor or to become a recipient itself. Among 10 Canadian spring wheat cultivars, Hucl (1996) observed that those with greater flower opening also exhibited the highest frequency of outcrossing. Wheat flowers generally remain open from 8 to 60 min depending on cultivar and environmental conditions (De Vries, 1971; 1973).

2.4.2 Stigma Characteristics and Receptivity

The stigma generally remains within the floret during pollination (Wilson, 1968). However, the stigmas of some cultivars have been observed to protrude beyond the edges of the glumes, providing increased opportunity for gene flow (De Vries, 1974b). This observation was recorded during a period of delayed pollination, but its discovery warrants further research both to underpin the cause and to quantify variation among current cultivars for this characteristic. Little is known regarding variation among wheat varieties for stigma length and the resulting influence on gene flow and whether gene flow is greater under conditions that provide for larger stigmatic surfaces and longer stigmas.

Duration of stigma receptivity is an important component affecting gene flow. Prolonged stigma receptivity enhances the likelihood of outcrossing and gene flow (De Vries, 1971). Stigmas appear to be most receptive for the first 2 to 5 d after anthesis (De Vries, 1971), but are reported to be receptive for 2 to 13 d following anthesis (Khan et al., 1973). This wide interval is likely a result of environmental conditions as stigma receptivity is optimal at moderate temperatures and relative humidity, similar to that required for optimal pollen shedding (De Vries, 1971). In fact, several studies have proposed that environmental effects are of greater significance than are genetic effects in affecting stigma receptivity (De Vries, 1971; Khan et al., 1973; Waines and Hegde, 2003).

2.4.3 Pollen Production and Viability

Pollen production and the factors affecting the viability of pollen have been well-studied. The number of pollen grains per anther varies among both wheat cultivars and among market classes. Joppa et al. (1968) reported from 2867 to 3867 pollen grains per anther among eight hard red spring wheat varieties. Likewise, De Vries (1974a) reported the mean number of pollen grains per anther to vary from 1746 to 2856 among five spring wheat varieties. The number of pollen grains produced per anther also appears to be a function of anther size. De Vries (1974a) observed that a range in anther length from

2.99 to 3.84 mm produced 1746 to 2856 grains per anther, with a correlation ranging from 0.43 to 0.75.

Generally, anther dehiscence results in 5-7% of pollen being shed on the stigma within the floret with 9-12% remaining on the anther (De Vries, 1971). Nevertheless, D'Souza (1970) estimated that as much as 30 to 80% of pollen was shed outside of the floret. This percentage can vary, however, depending on environmental conditions. A large amount of pollen is shed into the air under adverse conditions, and it is estimated that only one-third of this pollen falls within its own floret with the rest being available for cross-pollination (Leighty and Sando, 1924). Likewise, low moisture and high stress conditions often promote chasmogamous (flowers that open for pollination) flowering and increased outcrossing, resulting in a higher potential for gene flow (Waines and Hegde, 2003). Thus, pollen densities outside the floret can be such that insufficient isolation distances or favourable environmental conditions could be conducive to pollen-mediated gene flow (PMGF).

Anther extrusion plays an important role in cross-pollination and gene flow. Extrusion occurs as a result of filament elongation, which generally occurs over a period of 2 to 4 min (De Vries, 1971). The degree of floret opening can influence anther extrusion, with higher numbers extruded when the angle of separation between the palea and the lemma is greatest or when the duration of glume opening is longest (De Vries, 1971). Environmental conditions can likewise influence the number of extruding anthers. Livers (1964) reported the lowest percentage of seed set on male-sterile lines under drought conditions. Warm weather and high humidity caused rapid anther dehiscence and lower (45 to 50%) fertility compared to over 65% fertility under moderate temperatures and humidity (De Vries, 1971). Further, De Vries (1972) found that most pollen was released at temperatures between 16 to 20°C and relative humidity of 70 to 75%. Genotype can also reduce the percentage of extruding anthers, with percent extruding anthers of 25% to 72% for cultivars Centana and Thatcher, respectively (Joppa et al., 1968). Similarly, anther extrusion ranged from less than 30% to more than 80% among 10 wheat varieties (D'Souza, 1970).

Pollen is released from the tip of the extruded anther. Once attached to a stigma surface, pollen tube growth is initiated within about 1-2 h, with fertilization occurring 30-40 h later (De Vries, 1971). Wheat pollen is reported to be viable only for a short period of time (Wilson, 1968; D'Souza, 1970; De Vries, 1971; Major, 1980). Optimal viability under field conditions (i.e. approx. 0.5 h) occurs at warm temperatures (20°C) and moderate relative humidity (60%) (D'Souza, 1970; De Vries, 1971). Cool temperatures tend to reduce the duration of pollen shed and high temperatures reduce both duration of pollen shed and pollen viability (D'Souza, 1970; Major, 1980). Although it was estimated that wheat pollen moves at only 19 cm sec^{-1} over 2 m and generally travels only 1 m in still air (D'Souza, 1970), light winds and humid weather that favor pollen movement will tend to promote PMGF (Dowding, 1987) at various distances from the source.

2.4.4 Pollen Movement

Few studies have examined pollen dispersal in hexaploid wheat and the factors affecting it. The concentration of pollen in the air at a given time is a function of 1) pollen production per anther 2) percent of anther extrusion and 3) the number of anthers per unit area (Joppa et al., 1968; Virmani and Edwards, 1983). Of these three, anther extrusion was found to have the greatest effect on pollen dispersal, with Joppa et al. (1968) able to predict pollen shedding capacity of a cultivar from percent anther extrusion, number of pollen grains per anther, and number of fertile florets $plot^{-1}$.

Studies examining the effect of distance on pollen dispersal have produced conflicting results. Several studies have shown that seed set on male-sterile plants was low at distances greater than 3 m from pollinator plants (Suneson and Cox, 1964; De Vries, 1971). Likewise, Jensen (1968) demonstrated 90% of pollen remained within 6 m of its source, but pollen grains were nevertheless detected at 60 m from their source. Pollen grains have also been readily detected at distances ranging between 20 to 50 m (Suneson and Cox, 1964; D'Souza, 1970; De Vries, 1971). In a Kansas study the average number of pollen grains detected at 48 m ranged from 33 to 48, with pollen still detected at 60 m, the limit of the study (Khan et al., 1973). A subsequent Kansas study demonstrated wheat

pollen can travel up to 1000 m (Virmani and Edwards, 1983). Little is known regarding pollen movement at large scales and more research is needed. For example, the role of pollinator source size in influencing pollen movement at large distances is still relatively unknown (Willenborg and Van Acker, 2006). It is likely that a large pollinator source will result in increased detection of pollen at greater distances, especially given that gene flow in wheat has been reported to be directly related to the wind-borne pollen load (Bitzer and Patterson, 1967). However, it must be noted that pollen movement does not necessarily result in gene flow and although pollen movement has been reported up to 1000 m, the actual role of this pollen in outcrossing is still unknown (Pickett, 1993b).

2.4.5 Flowering Duration and Synchrony

In order for crop-to-crop or volunteer-to-crop PMGF to occur, several conditions must be met to facilitate unintended gene transfer (Van Acker et al., 2004). The volunteers or non-transgenic crop must grow at a distance where pollen exchange could occur, both species must flower synchronously, and sufficient cross pollination must occur such that effective ovule fertilization results and seed development occurs. Finally, the cross-pollinated seed must possess the germination capacity and fitness level to establish and produce viable progeny (Van Acker et al., 2004).

The consequences of flowering synchrony for individual fitness can vary greatly depending on duration of flowering and the quantity and temporal distribution of flowers (Augspurger, 1981; Gross and Werner, 1983) and will be magnified in cases where gene flow confers a fitness advantage. Consequently, flowering phenologies in the agroecosystem have ecological relevance at a range of scales from individuals to populations. Flowering synchrony between cultivars or species has generally been studied to determine competition for pollinators, phenological compatibility, and the resulting ecological implications (Primack, 1980; Augspurger, 1981, 1983; Ollerton and Lack, 1998). Researchers only recently have begun examining flowering synchrony between crops and weeds. For example, Simard and Légère (2004) demonstrated that synchrony of flowering was a major determinant of gene flow between canola (*Brassica*

napus) and wild radish (*Raphanus raphanistrum* L.) and may have important implications for PMGF and transgene movement.

Information on the synchrony of flowering between wheat crops and volunteers does not exist in the literature. Given that a substantial portion of volunteer wheat emergence generally occurs early in the growing season (De Corby et al., 2007) and that wheat crops tend to flower later in the season and for as few as 3 to 4 days (Hucl, 1996), flowering between the two may be largely asynchronous, thus greatly minimizing the potential for transgene movement. However, emergence can also occur in multiple cohorts (Anderson and Nielsen, 1996), which could greatly affect flowering synchrony with the crop. Future studies are needed to determine the potential role of flowering phenology in transgene movement, as well as assess the potential overlap among crop varieties and volunteers.

2.5 Intraspecific gene flow

Cultivar purity should be relatively simple and easy to maintain in wheat because it is predominantly a self-fertilizing species. However, because its mating system allows for partial inter-mating, off-types attributable to outcrossing are identified every year (Prakash and Singhal, 2003; Hucl et al., 2004). As a result, maximum impurity tolerances in Canada of 0.01% (one off-type per 10 000 plants) in foundation or registered seed and 0.05% (five off-types per 10 000 plants) in certified seed have been established for all wheat classes except Canadian prairie spring wheat (Anonymous, 2005b).

Several studies have established that outcrossing in wheat varies with cultivar, distance from the pollen source, and environmental conditions (Martin, 1990; Hucl, 1996; Briggs et al., 1999; Matus-Cádiz et al., 2004). Outcrossing frequencies are generally assumed to be less than 1% (Hucl and Matus-Cádiz, 2001), but can range from 0.3% to frequencies as high as 10.6%, depending on the cultivars studied (Martin, 1990; Hucl, 1996; Lawrie et al., 2006). Martin (1990) observed outcrossing frequencies from 0.1 to 5.6% for individual cultivars within a given year in Kansas winter wheat, with a semi-dwarf experimental line exhibiting the highest average outcrossing frequency of 3.1%.

However, no significant correlation was found between outcrossing frequency and plant height. A similar study in Canada detected an average range of 0.22% (cv. Katepwa; Campbell and Czarnecki, 1987) to 4.64% (cv. Oslo; Graf et al., 1990) outcrossing at a 20 cm distance using purple-grained pollinator rows as a phenotypic marker (Hucl, 1996). Outcrossing among all 10 cultivars averaged over the 2 yr study was 0.88%. Although no single spike characteristic was significantly correlated with outcrossing frequency, higher outcrossing cultivars tended to have a greater degree of spikelet opening at anthesis. Lawrie et al. (2006) obtained up to 10.6% outcrossing between spring wheat cultivars under greenhouse conditions using the contact method, where pollinator (male) spikes were placed on source spikes (female) and covered with a glassine bag.

More recent studies have focused on assessing gene flow at larger scales, permitting more generalized conclusions regarding the potential for transgene movement in wheat. Hucl and Matus-Cádiz (2001) compared four wheat cultivars in Saskatchewan at distances ranging from 0 to 30 m and found that gene flow declined considerably with increasing distance from the pollen source, depending on cultivar and wind direction. Maximum gene flow at 30 m from the pollen source ranged from 0.2 to 3.8%. However, both the size (5 x 5 m) and density (60 plants m^{-2}) of the pollinator source was low. The authors concluded that isolation distances of at least 30 m are required for certain cultivars to reduce off-type levels in pedigreed seed. In a larger Saskatchewan field scale study using a 50 x 50 m pollinator block, maximum gene flow averaged over all directions was < 0.5% and decreased rapidly with increasing distance from the pollen source, although gene flow was confirmed at 300 m (Matus-Cádiz et al., 2004). Average intraspecific gene flow frequencies were higher and were confirmed at greater distances than interspecific (*T. turgidum*) gene flow. Elevated gene flow frequencies were also reported to be associated with the direction of the prevailing winds. However, the pollinator block was seeded at a low density (100 plant m^{-2}) and although flowering overlap was likely high due to multiple seeding dates, the pollen cloud produced would have been much reduced compared to that expected under field conditions. Extrapolation of these results to a commercial scale is precluded by the small size of the pollinator source and the fact that sampling intervals were not increased with increasing distance

from the pollen source, reducing the probability of detection at greater distances. Similar conclusions were drawn by Hanson et al. (2005b) and Gatford et al. (2006). Using a Nelder wheel design with a central pollinator source (0.16 ha), Hanson et al. (2005b) detected higher gene flow frequencies in the prevailing wind direction with a maximum gene flow frequency not exceeding 0.45% in winter wheat in the Pacific Northwest region of the USA. The authors failed to detect gene flow beyond 42 m and therefore recommended an isolation distance of 45 m to prevent PMGF. Likewise, Gatford et al. (2006) reported levels of gene flow of 0.012% and 0.0037% at two locations in Australian winter wheat; gene flow was not detected beyond 12 m.

Based on data from previous studies, Gustafson et al. (2005) developed an empirical model which demonstrated that even in the absence of buffer zones, gene flow in harvested-blended (harvested grain from entire field blended together) wheat would be < 0.1%, well below any labeling thresholds. However, Willenborg and Van Acker (2006) contend that there are serious problems with both the approach taken and conclusions drawn from the model results. The model was parameterized with data from very small pollen sources (< 1 ha), and erroneously concluded that pollen source sizes greater than 10 ha were not important to gene flow. Furthermore, the model predicted that little gene flow would exist beyond 100 m. To the contrary, a recent commercial-scale study of PMGF in wheat detected gene flow as far as 2.75 km from pollinator sources of 33 and 20 ha (Matus-Cádiz et al., 2007). While frequencies of gene flow detected by Matus-Cádiz et al. (2007) were low (< 0.01%), the same logistical difficulties noted above precluded a complete assessment of potential gene flow and the true level of outcrossing may be higher than that reported by Matus-Cádiz et al. (2007). While it is likely that PMGF will be less consequential to admixture than seed movement, gene flow through seed movement could theoretically be prevented and/or contained whereas PMGF cannot. Considering PMGF is only one of several vectors contributing to product admixture, levels of gene flow < 0.01% should not be disregarded as inconsequential, particularly given the effects of possible further selection pressure for some traits (Brûlé-Babel et al., 2006).

2.6 Interspecific hybridization and introgression with wild relatives

The sexual transfer of transgenic traits from a crop to a weed is the primary concern in developing and releasing a transgenic crop (Goodman and Newell, 1985). Most crop species have wild relatives and natural hybridization is known to occur (Ellstrand and Hoffman, 1990; Ellstrand et al., 1999). Introgression refers to trait stabilization produced from repeated backcrossing of hybrid progenies with their parents. Successful introgression initially requires the formation of fertile hybrids and backcross (BC) individuals. Introgression may be affected by several factors including ploidy level of the species, genetic relatedness between them, and the direction of hybridization (Rieseberg and Wendel, 1993). In North America, hexaploid wheat has two wild weedy relatives to which it may cross, resulting in hybridization and potential introgression (Hegde and Waines, 2004). These are jointed goatgrass (*Aegilops cylindrica* Host.) and feral rye (*Secale cereale*), both of which have not yet been reported in Canada (although annual rye crops are a component of western Canadian cropping systems). Gene flow could also conceivably occur between triticale (X *Triticosecale* Wittmack) and wheat crops. Spontaneous crossing between wheat and rye lead to the development of triticale, which is a man-made hybrid of wheat and rye bred in the late 19th century. Although potential for PMGF between wheat and triticale exists where triticale is grown for seed or ethanol production, triticale is more commonly harvested as silage or greenfeed before seed maturation occurs.

2.6.1 Hybridization with *Aegilops cylindrica*

Jointed goatgrass is a winter annual that is primarily a weed of fall-sown or winter wheat, but can occur in other cereal grains and in disturbed non-crop areas (Donald and Ogg, 1991). Jointed goatgrass (allotetraploid, CCDD genomes, $2n = 4x = 28$ chromosomes) and wheat share the D genome contributed to each by a common ancestor, *Aegilops tauschii* (Kimber and Sears, 1987), and hybrids have been reported under natural conditions (Seefeldt et al., 1998; Zemetra et al., 1998; Stone and Peeper, 2004). Moreover, naturally occurring hybrids ($2n = 35$) have been shown to produce viable,

fertile seed. Seed set on jointed goatgrass x wheat hybrids under field conditions averaged 2.3% and 3.8% in yr 1 and yr 2, respectively, with the germination rate being 94% for both years (Snyder et al., 2000). In the same study, an average of 3.1% of BC_1 (first backcross generation) plants (produced in the field) set seed under greenhouse conditions. A similar greenhouse study reported seed set on BC_1 plants was similar regardless of the recurrent parent, whereas female fertility increased from 2% in the hybrid to 37% in BC_2 plants with jointed goatgrass as the recurrent parent (Zemetra et al., 1998). Wang et al. (2001) concluded that seed produced on hybrid plants is not likely the result of self-pollination, but rather, hybrids are generally male-sterile with a low level of female fertility. Therefore, fertilization must occur either by wheat or jointed goatgrass pollen (Wang et al., 2001). Partial self-fertility was restored in a few select plants by BC_2, and self-fertility increased rapidly with increasing BC generations (Wang et al., 2001).

Field studies have also confirmed natural hybridization between *T. aestivum* and *A. cylindrica*. Two imazamox-resistant hybrids were discovered in research plots in Washington where FS4 imidazolinone-resistant wheat from a previous imazamox efficacy trial conducted the year prior was replanted (Seefeldt et al., 1998). These hybrid plants survived normally lethal imazamox applications and subsequently produced seven viable seeds in the BC_1 generation (backcrossed to wheat). While this is the only case to date of natural hybrids having herbicide resistance, Morrison et al. (2002) also observed naturally occurring hybrids in Eastern Oregon, where 45% of the naturally-occurring hybrid plants had one or more fertile spikes, with an average of 1.4 viable seeds $spike^{-1}$. These results support the possibility of transgenes moving from wheat to hybrids and eventually to jointed goatgrass populations under natural conditions via hybridization. Moreover, resistance to imazamox herbicide is conferred by the gene on chromosome 6D (the genome shared with jointed goatgrass) in some wheat cultivars resistant to imazamox (Anderson et al., 2004). Furthermore, resistant jointed goatgrass x wheat hybrids have been produced up to 40 m from a wheat pollen source with outcrossing frequencies ranging between 0.04 to 0.5% (Hanson et al., 2005a).

Introgression of genetic material from hexaploid wheat into *A. cylindrica* can occur via three mechanisms. The most frequent is recombination of the homologous D

chromosomes and even though the D genome of wheat and *A. cylindrica* originated from different biotypes of *A. tauschii*, they pair successfully at meiosis forming 14 bivalents (Zemetra et al., 1998; Badaeva et al., 2002). The second method is through intergenomic translocations or chromosome rearrangements. Wang et al. (2000) demonstrated that a translocation from the A or B genome of wheat to *A. cylindrica* had occurred in a BC_2S_2 plant. Finally, introgression can occur through disomic chromosome retention. Schoenenberger et al. (2005) identified an individual having 31 chromosomes (normal $2n = 35$) meaning that either there was one A or B genome chromosome present in a homologous pair. Alternatively, there may have been three different A or B genome chromosomes present as monosomes, which presupposes the presence of a substituted chromosome in the BC_1 plant. Although it has been suggested that placing traits on either the A or B genomes prevents introgression into jointed goatgrass, Schoenenberger et al. (2005) demonstrated that successful introgression of wheat A and B genome fragments into *A. cylindrica* can occur. In fact, the authors conclude that it is not sufficient to place a transgene on those genomes to effectively avoid its introgression.

These previous studies clearly indicate that PMGF can result in potential movement of a herbicide resistance trait or transgene from wheat to jointed goatgrass. Introgression may subsequently occur and the risk that a transgene located on either the A or B genome of wheat is maintained in the *A. cylindrica* background depends on the frequency of chromosome retentions and translocations for each genomic and chromosomal region of wheat (Schoenenberger et al., 2005). Should the transgene be one that confers a selective advantage, such as herbicide tolerance, it could result in the entire population of jointed goatgrass rapidly acquiring the trait in a very short period of time. The length of time required would be a function the selection pressure imposed as well as the fitness advantage, if any, conferred by the newly acquired trait. While several studies have focused on gene flow between wheat and jointed goatgrass, little is currently known about flowering duration, phenology, and synchrony of jointed goatgrass with wheat. In addition, the fate of transgenes in jointed goatgrass remains largely unstudied.

2.6.2 Hybridization with *Secale cereale*

Rye is a commonly grown cereal crop in many regions of North America, but also exists as a feral weed in the Midwestern United States (Burger and Ellstrand, 2005). Globally, it appears that natural introgression between hexaploid wheat and rye is uncommon (Hegde and Waines, 2004). The ability of wheat and rye to cross is believed to be controlled by dominant genes in rye (Tanner and Falk, 1981). Despite the apparent inability of rye x wheat hybrids to occur under natural conditions, artificial crosses between the two have successfully produced hybrids (Falk and Kasha, 1981).

Wheat x rye hybrids are generally male sterile and highly female fertile in most cases (Maan, 1987). Moreover, wheat x rye hybrids are easier to produce than are rye x wheat hybrids (Leighty and Sando, 1928). Fertilization involving unreduced functional gametes occasionally results in selfed seed able to produce fertile amphiploid progeny (Maan, 1987). The chromosomes of *Triticum* ssp. do not pair with chromosomes of *Secale* ssp., and meiosis in the F_1 generation is highly irregular. The occurrence of rye x wheat hybrids with introgressed genes from wheat thus appears highly unlikely, particularly under natural conditions. Nevertheless, Hegde and Waines (2004) conclude that it is entirely possible that interactions between the pairing and crossing of genes in wheat and rye may either reduce or possibly enhance seed set, depending on the hybridization direction. However, little research in the last 80 yrs has examined the potential for and the conditions required to facilitate hybridization. Given that rye is grown on over 6 Mha worldwide (FAOSTAT, 2006), introgression of a transgene into the population could have important consequences. A low level of dormancy can be induced in feral rye (Stump and Westra, 2000), which may contribute to its feral nature. This dormancy mechanism could be important in the temporal persistence of introgressed traits and transgenes.

3.0 Response of heterozygous and homozygous imidazolinone-resistant spring wheat genotypes to imazamox

3.1 Introduction

Wheat is grown on over 30 million hectares in North America (FAOSTAT, 2008). Many weeds, particularly annual grasses and volunteer cereal crops, are difficult to selectively control in wheat crops. The development of herbicide-resistant (HR) crop cultivars, which now exist for most of the major world crops (Devine, 2005), has resulted in improved selective weed control in many crops, including wheat. Cultivars of wheat possessing resistance to imidazolinone (IMI) herbicides have been released for wide-scale cultivation in the United States and Canada. Imidazolinone-resistant (IR) winter wheat was released commercially in the Great Plains and Pacific Northwest regions of the United States in 2002 and 2003, respectively (Hanson et al., 2007). Likewise in Canada, IR spring wheat (cv. CDC Imagine) was released in 2004 and was the first HR wheat cultivar grown commercially in Canada. These IR wheat cultivars possess tolerance to the imidazolinone class of herbicides that inhibit acetohydroxyacid synthase [AHAS; also known as acetolactate synthase (ALS)], the first enzyme catalyzing the biochemical synthesis of the branched chain amino acids valine, leucine, and isoleucine (Shaner et al., 1984; Singh, 1999). Imazamox is registered for use on both winter and spring IR wheat because of its broad weed control spectrum, limited soil persistence, and favorable toxicological properties (Shaner et al., 1996; Tan et al., 2005). Adoption of these cultivars has been rapid as IR winter wheat was grown on 242,000 ha in the United States in 2006 (Hanson et al., 2006) while 121,500 ha were planted to IR spring wheat in western Canada in 2007 (Kristin Hacault, BASF Canada Inc., personal communication).

Imidazolinone-resistant wheat was originally developed through seed mutagenesis of cv. Fidel winter wheat followed by screening with the imidazolinone herbicide imazethapyr (Newhouse et al., 1992). Surviving wheat plants all possessed a single, semi-dominant nuclear gene coding for imidazolinone-resistant ALS (Newhouse et al., 1992; Pozniak and Hucl, 2004) located on chromosome 6DL (Seefeldt et al., 1998; Anderson et al., 2004; Pozniak et al., 2004a). Subsequent work confirmed the presence of three homoeologous ALS genes in wheat located on chromosomes 6AL, 6BL, and 6DL, each contributing approximately one-third of the total resistant ALS (Pozniak and Hucl, 2004; Pozniak et al., 2004a; Hanson et al., 2006). The mutation on chromosome 6DL was named *Imi1*, while mutations on the A and B genomes subsequently induced in cv. CDC Teal (Hughes and Hucl, 1993) lines derived from mutagenesis have been named *Imi3* and *Imi2*, respectively (Pozniak and Hucl, 2004). Physiological and molecular characterization of the mutations revealed that both *Imi1* and *Imi2* possess a Ser_{653}-Asn_{653} amino acid substitution relative to the *Arabidopsis thaliana* L. Huynh. consensus sequence (Pozniak et al., 2004a). Because IR cultivars were derived through mutagenesis, they are not considered transgenic and adventitious presence of this trait is not a concern to wheat buyers (Demeke et al., 2006).

Hexaploid wheat is the most prevalent crop grown for and directly consumed by humans in which transgenic cultivars are under development (Braun et al., 1998). Concerns over market acceptance of transgenic wheat and the importance of protecting patented plant cultivars necessitates heightened attention around the issue of crop to crop and volunteer to crop gene flow (Conner et al., 2003; Kershen, 2004). Gene flow in wheat can be facilitated by seed commingling or pollen exchange; in both cases, movement of the gene is often traced with phenotypic markers. While several studies have utilized the blue aleurone trait to examine gene flow (Hucl and Matus-Cádiz, 2001; Matus-Cádiz et al., 2004; Matus-Cádiz et al., 2007), others have utilized IR wheat as a phenotypic marker (Hanson et al., 2005a; Gaines et al., 2007a, 2007b). Imidazolinone-resistant wheat should be an ideal candidate trait for measuring gene flow because it provides a distinguishable marker, with hybrid plants exhibiting an intermediate phenotype between R and S (dead) control plants when exposed to a normally lethal dose

of imazamox (Pozniak and Hucl, 2004). In addition, IR wheat provides a commercially available phenotypic marker with which gene flow can be examined between wheat crops. However, because hexaploid wheat contains three diploid genomes (A, B, D) and ALS resistance in wheat is produced by a multigene family, the enzyme produced by the A and B genome homoeologues in current Canadian IR wheat (cv. CDC Imagine) cultivars is not resistant to IMI herbicides (Pozniak et al., 2004a). This can result in variable resistance when the IR trait is conferred by a single gene, occasionally resulting in plant injury (Pozniak et al., 2004b). Moreover, factors such as doses, surfactants, and application timing can strongly influence the level of crop injury (Geier et al., 2004; Stougaard et al., 2004; Frihauf et al., 2005).

In order for IR spring wheat to be used accurately as a phenotypic marker in field studies examining gene flow, dose-response analysis and phenotypic characterization of the trait at the whole-plant level should be carried out. While it has been reported that D-genome IR wheat biotypes are more resistant to imazamox than B-genome IR biotypes, and winter IR biotypes more resistant than spring (Hanson et al., 2006), the effects of parental background are less clear (Pozniak et al., 2004b; Rainbolt et al., 2005; Hanson et al., 2006). Hanson et al. (2006) demonstrated that parental background did not influence ALS tolerance in homozygous IR spring and winter wheat lines. Among spring wheat lines, BW755 exhibited greater tolerance to imazamox than TealIMI 10A, suggesting that varietal differences may affect the level of field resistance of spring wheat to imazamox (Pozniak et al., 2004b). Rainbolt et al. (2005) also suggested that parental background or market class may affect IR wheat ALS sensitivity to imazamox, but their results were inconclusive. Furthermore, near-isogenic lines of IR spring and winter wheat also exhibited differences in the recovery of ALS activity following imazamox application (Hanson et al., 2007). Information from phenotypic characterization and dose-response studies are necessary to determine the suitability of the IR trait as a phenotypic marker in wheat gene flow studies, which often include multiple lines or cultivars. If parental background differences exist, each line or genotype would potentially have to be screened at different imazamox doses to reliably detect hybridization events. However, no published information exists regarding the response of heterozygous IR spring wheat

genotypes differing in parental background to various doses of imazamox. Therefore, the objective of this research was to compare the sensitivity of several spring wheat genotypes of different parental backgrounds and market classes to a range of doses of imazamox at the whole-plant level.

3.2 Materials and Methods

3.2.1 Parental and F_1 Populations

Commercial pedigreed (certified) seed for the selected spring wheat genotypes was obtained from their respective seed distributors. All wheat seeds used in the parental population were from commercially available genotypes, which were chosen on the basis of diversity in pedigrees and market class groupings. Susceptible genotypes included two Canada western red spring (CWRS) (cv. AC Barrie and cv. Prodigy), two Canadian prairie spring (CPS) (cv. Oslo and cv. AC Vista), and one Canada western extra strong (CWES, cv. Amazon) (Table 3.1). CDC Imagine (CWRS), an IR-wheat genotype that has a single semi-dominant nuclear gene encoding for resistance on chromosome 6DL (Pozniak and Hucl, 2004; Pozniak et al., 2004a), was included as a standard and used as the male parent in generating F_1 hybrids.

To produce F_1 seed, parental plants were grown in a growth room with a 16-h photoperiod, 21/15°C day/night temperature, and an approximate radiance of 480 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Three seeds were initially planted in 15 cm diameter (3.75 L) pots filled with commercial potting media (Sunshine Mix no. 3, SunGro Horticulture, Bellevue, WA) and were thinned to one plant per pot at the two leaf stage. Plants were watered as required based on visual observation of the soil surface until the two leaf stage after which plants were watered daily. Plants were fertilized weekly with a dilute solution of water-soluble fertilizer (Peters Professional Water Soluble Fertilizer 20-20-20, W. R. Grace & Co., Fogelsville, PA) at the recommended rate of 2.4 g L^{-1} product. To ensure synchronous anther dehiscence and sufficient pollen

TABLE 3.1 Parental information, resistance trait expression, market class, and wheat genotypes used in the imazamox dose-response experiment.

Genotype	Market class†	Resistance trait genotype‡	Parental Pedigree	Reference
CDC Imagine	CWRS	(H) Resistant	CDC Teal*4/Fidel(FS2)	P. Hucl, pers. comm.
Amazon	CWES	(H) Susceptible	Glenlea*6/Salamouni	Anonymous (2005c)
AC Barrie	CWRS	(H) Susceptible	Neepawa/Columbus//BW90	McCaig et al. (1996)
Oslo	CPSR	(H) Susceptible	Sonora 64/Yaqui 50E//Guajalote/3/Inia/4/ Ciano//Elgan/Sonora 64	Graf et al. (1990)
Prodigy	CWRS	(H) Susceptible	SWP 2242/Stoa	Graf et al. (2003)
AC Vista	CPSW	(H) Susceptible	HY344/Losprout 'S'//HY358*3/BW553	DePauw et al. (1998)
Amazon/CDC Imagine	-	Heterozygous	-	-
AC Barrie/CDC Imagine	-	Heterozygous	-	-
Oslo/CDC Imagine	-	Heterozygous	-	-
Prodigy/CDC Imagine	-	Heterozygous	-	-
AC Vista/CDC Imagine	-	Heterozygous	-	-

†Abbreviations: CPSR, Canada prairie spring red; CPSW, Canada prairie spring white; CWES, Canada western extra strong; CWRS, Canada western red spring.
‡Homozygous (H), heterozygous, or susceptible single gene imidazolinone resistance (D genome).

availability for crossing, three planting dates were used for all parent plants, with one planting per week for a period of three weeks. All florets on five spikes of maternal plants were emasculated prior to anther maturation and when the stigma was receptive, pollinated with CDC Imagine pollen. To produce enough F_1 seed for the dose-response experiments, emasculation and crossing among the three plantings occurred over a period of 42 d during which individual stigmas remained receptive for approximately 10 d. Seeds were harvested at maturity and stored briefly at room temperature prior to use. According to accepted convention, F_1 genotypes are designated with the maternal plant preceding the pollen donor (Table 3.1).

3.2.2 Dose-Response Experiment

Four wheat seeds of each genotype (Table 3.1) were planted in commercial potting media in 11 cm-diam (1 L) pots, and were thinned to three plants per pot at the two leaf stage. The experiment was arranged as a completely randomized design with two experimental runs (separated in time) and three replications per treatment (one pot per replicate). All pots were initially watered to field capacity with a daily watering regime commencing at the one leaf stage. All other growing conditions were as described previously for the production of F_1 seed. At Haun stage 2.5 to 3.0 (Haun, 1973), R and F_1 pots were randomly assigned to seven imazamox (Solo herbicide, BASF Canada Inc., Toronto, ON) doses (0.4, 1.3, 3.9, 11.7, 35, 105, 315 g a.i. ha^{-1}), an untreated control, and a zero-time shoot biomass determination. Susceptible plants were sprayed with doses ranging from 0.4 to 11.7 g a.i. ha^{-1}, because previous indoor trials had indicated that complete kill of susceptible wheat seedlings occurred at approximately 10 g a.i. ha^{-1} of imazamox (Hanson et al., 2006). The recommended field dose of imazamox for IR spring wheat in Canada is 20 g a.i. ha^{-1} (Anonymous, 2008). Imazamox treatments were applied with a cabinet sprayer equipped with a flat fan nozzle (TeeJet SS80015 flat fan nozzle tip, Spraying Systems Co., Wheaton, IL) calibrated to deliver 117 L ha^{-1} of spray solution at 310 kPa in a single pass over the foliage, with an adjuvant (Merge adjuvant, BASF Canada Inc., Toronto, ON) added at 0.5% (v/v) to the spray solution. To determine shoot

biomass at the time of herbicide application, 9 plants (3 pots) of each R, F_1, and S genotype were clipped at the soil surface, oven dried at 80°C for 48 h, and weighed. Twenty-one days after treatment (DAT) all plants were visually assessed as dead (absence of new green leaves), or alive, aboveground shoot biomass was harvested, and per-plant dry weight was determined.

3.2.3 Statistical Analysis

To determine the influence of imazamox on shoot growth, shoot biomass from each wheat genotype at the time of herbicide application was determined by subtracting the appropriate zero-time shoot biomass from final shoot biomass for each sample. Residuals for all data were initially tested for homogeneity of variance and normality to ensure data conformed to the assumptions of ANOVA. Residuals deviated slightly from a normal distribution but several transformations failed to improve normality; therefore, untransformed data were used in ANOVA. To determine if shoot biomass accumulation prior to imazamox application differed among wheat genotypes, zero-time shoot biomass data were subjected to a one-way analysis of variance using the mixed model procedure of SAS (Littell et al., 1996; SAS Inst., 2004), with degrees of freedom calculated by Satterthwaite's approximation method. Experimental run and its interaction with fixed effects were considered random in the model whereas wheat genotype was considered fixed. To determine whether data could be combined across runs, a log-likelihood ratio was utilized to test the significance of the random effect of run and its interactions with fixed effects (Littell et al., 1996). Means were separated using Fisher's protected LSD with treatment effects declared significant at $P < 0.05$.

Prior to nonlinear regression analysis, shoot biomass data were converted to a percentage of the untreated control plants within each genotype to allow for direct comparisons between groups. Statistical analysis of dose-response curves closely followed the procedure outlined by Seefeldt et al. (1995). Data were fit to a log-logistic model given by:

$$y = C + \frac{D - C}{1 + \left(\frac{x}{GR_{50}}\right)^{b}} \quad (3.1)$$

where y = shoot biomass (expressed as a percent of the appropriate untreated control), x = imazamox dose (g a.i. ha^{-1}), C = lower asymptote, D = upper asymptote, b is a rate parameter (slope) related to the response to increasing imazamox dose, and GR_{50} is the imazamox dose that caused a 50% reduction in shoot biomass accumulation. Regressions were performed on all data using a derivative-free, nonlinear least squares regression procedure (PROC NLIN; SAS Inst., 2004). Adequacy of model fit was determined by significance of the model approximate F statistic, coefficients of determination (*pseudo* R^2), and examination of the underlying residual structure. Individual curves were statistically tested systematically for common C, D, b, and GR_{50} parameters using the lack-of-fit F-test procedure at the $P < 0.05$ significance level, as outlined by Seefeldt et al. (1995). Coefficients of determination were calculated by using the residual sum of squares value from paired model estimates as suggested by Kvalseth (1985). As detailed by Seefeldt et al. (1995), SAS provides only one residual sum of squares value for the model as a whole, even though parameters for several curves are estimated concurrently. Standard errors of the parameter estimates are presented; the standard error of a parameter estimate is a measure of confidence and if it is greater than 50% of the numerical value of the estimate, the parameter is considered poorly estimated (Koutsoyiannis, 1977).

3.3 Results

Analysis of variance indicated no significant ($P < 0.05$) effect of experimental run on any of the data; therefore, data from the two experiments were combined for further analysis. Initial examination of individual dose-response curves for the 11 genotypes included in this study revealed three distinct groups of curves (Fig. 3.1). The three groups consisted of the five S genotype response curves grouped closely together, the five F_1

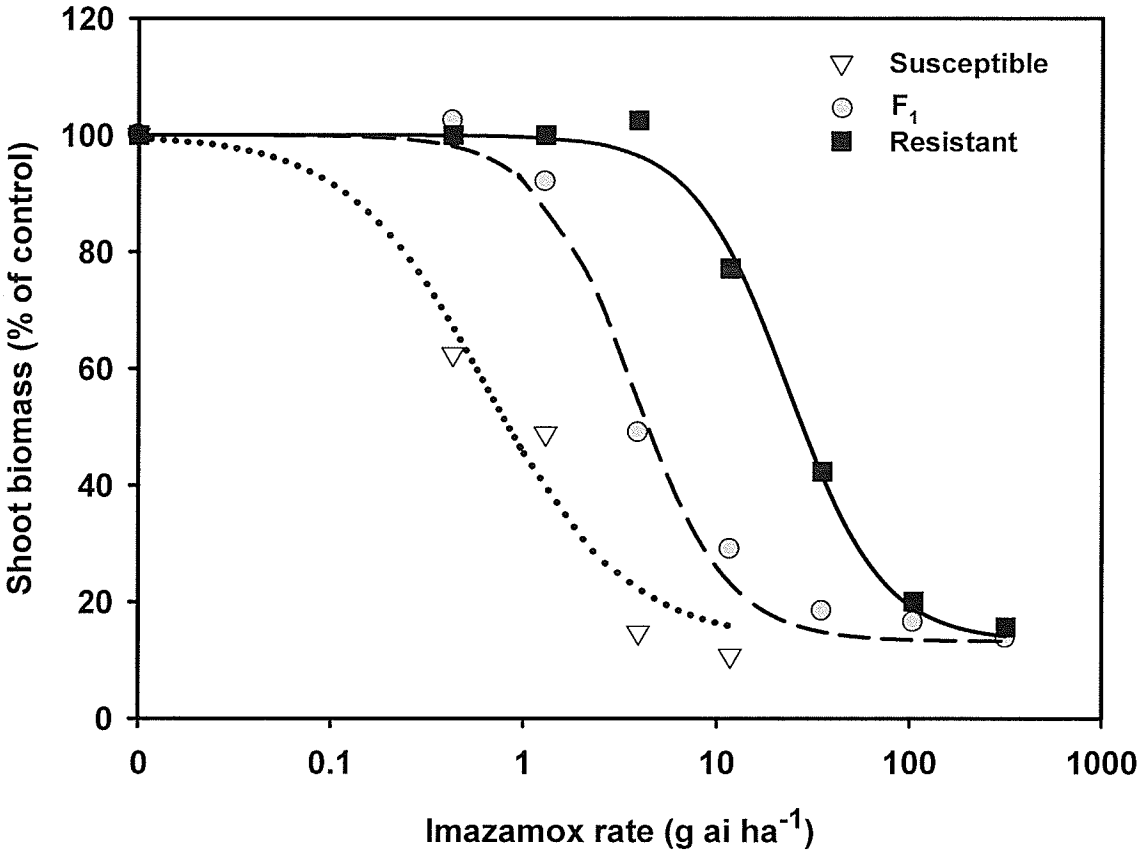

FIGURE 3.1 Response of parental and F_1 spring wheat genotypes to foliar applied increasing imazamox dose as measured by shoot biomass accumulation 21 DAT. Mean shoot biomass (% of control) values are represented by the symbols, while predicted values ($R^2 = 0.99$) are represented by the lines. Means of the S and F_1 groups are pooled averages of the five genotypes within that group. Refer to materials and methods for a description of statistical procedures.

genotype curves grouped closely together, and the single homozygous IR genotype (CDC Imagine) response curve by itself. Lack-of-fit *F*-tests confirmed that the five S genotype response curves could statistically be combined into a single curve; likewise, the five F_1 genotype curves were combined into a different single curve. This indicates that in this study, neither parental background nor market class grouping significantly affected spring wheat response to imazamox.

The log-logistic model satisfactorily described shoot biomass accumulation following imazamox application for all wheat genotypes, as indicated by the significance of approximate *F*-tests ($P < 0.001$) and an overall model R^2 value of 0.99 (Fig. 3.1, Table 3.2). Residual analysis revealed that the errors conformed to the underlying regression assumptions (data not shown). Because shoot biomass data were expressed as a percentage of the appropriate untreated control plants within experiments and genotypes, the upper response asymptote parameter (*D*) was held constant at 100% in all instances during curve fitting. The lower asymptote parameter (*C*), the rate parameter (*b*), and the GR_{50} parameter were all allowed to vary among R, S, and F_1 curves. Lack-of-fit *F*-tests indicated that the three curves shared the same lower asymptote parameter (*C*), while the R and F_1 curves were also found to have a common slope parameter (*b*) (Table 3.2). This is not unexpected as Streibig et al. (1993) proposed that parallel dose-response curves suggest that the herbicide is acting at the same site of action for each R genotype (i.e. F_1 and homozygous R have site-of-action based resistance) and that the affinity for the herbicide at its site of action in the homozygous R genotype was reduced. The *b* parameter value was significantly lower for the S genotypes than the R and F_1 genotypes. Little reduction in shoot dry matter was observed in the R genotype and F_1 genotypes at doses below 1.3 and 3.9 g a.i. ha^{-1}, respectively (Fig. 3.1). The S genotype, on the other hand, exhibited a 38% decline in shoot biomass at 0.4 g a.i. ha^{-1}, the lowest imazamox dose used in the study.

Model GR_{50} values varied substantially among the three genotype groups, ranging from 0.65 to 23.2 g a.i. ha^{-1} for S and R genotypes, respectively (Table 3.2). The GR_{50} value of the F_1 genotypes was significantly greater than that of the S genotype, indicating that crosses made among parental genotypes resulted in F_1 progeny with a six-fold increase in

TABLE 3.2 Model parameter estimates, standard errors, and R/S ratio estimated by non-linear regression for shoot biomass accumulation by spring wheat genotypes in response to increasing doses of foliar imazamox.

Wheat genotype	Parameter†,‡	Estimate (standard error)	GR_{50} ratio R/S
CDC Imagine (R)	GR_{50}	23.20 (2.52)	36
	b	1.76 (0.21)	
	C	13.25 (1.88)	
F_1	GR_{50}	3.67 (0.39)	6
	b	1.76 (0.21)	
	C	13.25 (1.88)	
Susceptible (S)	GR_{50}	0.65 (0.09)	NA§
	b	1.20 (0.23)	
	C	13.25 (1.88)	

†The upper asymptote parameter, *D*, was set to 100% for all wheat genotypes.
‡Abbreviations: *C*, lower response asymptote; *D*, upper response asymptote; *b*, a rate-related (slope) parameter; GR_{50}, imazamox dose (g a.i. ha^{-1}) causing a 50% reduction in shoot biomass accumulation. Refer to materials and methods for a description of the model fitted.
§Not applicable.

resistance compared to the S genotypes. These differences were not due to initial shoot biomass accumulation prior to imazamox application.

Phenotypic mortality characterization of the response of IR spring wheat to imazamox demonstrated a consistent effect at low and high doses, but variability existed at intermediate doses (Table 3.3). At low doses (0 to 1.3 g a.i. ha^{-1}) plant survival among all genotypes was 100%, while at high doses (35 to 105 g a.i. ha^{-1}) no plants survived among F_1 genotypes, with only the homozygous R genotype (CDC Imagine) surviving. An intermediate dose of 3.9 g a.i. ha^{-1} resulted in the survival of 100% of R and F_1 genotypes, but 39% and 6% of S Amazon and Vista plants also survived imazamox application, respectively. Although the GR_{50} value of F_1 genotypes occurred at approximately this dose, failure to achieve 100% mortality in some S genotypes indicates higher doses are necessary to discriminate accurately between F_1 and S plants if IR wheat is used as a phenotypic marker in PMGF studies. At 11.7 g a.i. ha^{-1}, 100% of R and F_1, and 0% of S genotypes survived the foliar imazamox application. Visual differences were very apparent at this dose with F_1 genotypes exhibiting an intermediate phenotype between R and S parental phenotypes (Fig. 3.2). Thus, 12 g a.i. ha^{-1} should be an appropriate indoor discriminating dose of imazamox where IR spring wheat is used as a phenotypic marker in gene flow studies.

3.4. Discussion

Despite observing greater than two-fold variation in zero-time shoot biomass accumulation data, we did not detect significant differences between genotypes or groups of genotypes (data not shown). This is congruent with Pozniak et al. (2004b) who also did not detect significant differences in growth parameters between mutagenized IR spring wheat lines derived from cv. CDC Teal and their susceptible parent (CDC Teal). Based on our results, resistance to imazamox among wheat genotypes was not a function of plant size prior to imazamox application, but was due to the production of resistant ALS. Approximately 37% of the ALS enzyme in spring wheat plants was resistant to in vitro imazamox application when the resistance gene was located on the D-genome

TABLE 3.3 Percentage of wheat plants surviving increasing imazamox doses, as visually assessed 21 DAT. Standard errors are presented in parentheses where variation in the response was observed.

	Imazamox dose (g a.i. ha^{-1})							
Wheat genotype†	0	0.4	1.3	3.9	11.7	35	105	315
	%							
Imagine (R)	100	100	100	100	100	100	56 (27)	0
Amazon (F_1)	100	100	100	100	100	0	0	0
Barrie (F_1)	100	100	100	100	100	0	0	0
Oslo (F_1)	100	100	100	100	100	0	0	0
Prodigy (F_1)	100	100	100	100	100	0	0	0
Vista (F_1)	100	100	100	100	100	0	0	0
Amazon (S)	100	100	100	39 (49)	0	NA‡	NA	NA
Barrie (S)	100	100	100	0	0	NA	NA	NA
Oslo (S)	100	100	100	0	0	NA	NA	NA
Prodigy (S)	100	100	100	0	0	NA	NA	NA
Vista (S)	100	100	100	6 (13)	0	NA	NA	NA

†All F_1 genotypes are the result of hand-crossing to CDC Imagine.
‡Not applicable.

FIGURE 3.2 The three distinct plant response phenotypes observed 21 DAT following treatment with 12 g a.i. ha^{-1} imazamox. From left to right, homozygous resistant (R), heterozygous intermediate (F_1), and homozygous susceptible (S) plants.

(Hanson et al., 2007), as was the case in our study. In a similar study, Hanson et al. (2006) reported that in vitro applications of 50 μmol L^{-1} imazamox resulted in approximately 35.7% ALS activity remaining in the D-genome wheat extract and suggested that the D-genome accounts for slightly more total ALS activity than either the A- or B-genomes in both spring and winter wheat.

With respect to imazamox activity on spring wheat, results from this trial do not correspond directly with field observations, but they do provide some interesting observations. Under controlled environment conditions, growth of homozygous IR spring wheat (CDC Imagine) was greatly reduced at the registered field dose of imazamox (predicted 38% reduction in shoot biomass accumulation at 20 g a.i. ha^{-1}; Fig. 3.1). This suggests that imazamox is more active indoors than under field conditions. However, in the field, plant injury and stunting may not persist throughout the season to negatively affect grain yield (Pozniak et al., 2004b). Both the number and location of resistance genes are known to affect the response of IR wheat to imazamox (Pozniak et al., 2004a; Hanson et al., 2006). Commercially released spring wheat genotypes as well as the genotypes in Canada used in this study possess resistance on the D genome, which contains 10% more total in vitro ALS activity in the presence of imazamox compared with resistance located on the B genome (Pozniak et al., 2004a; Hanson et al., 2006). Two resistance genes may be necessary to improve crop safety and yield with currently registered imazamox doses. Pozniak et al. (2004a) developed a line (Teal 15A) that is reported to be homozygous for two nuclear dominant IMI resistance genes. The additive level of resistance conferred by a two-gene system (AD genomes) provides increased levels of whole-plant resistance (Pozniak et al., 2004b; Hanson et al., 2006) and is currently being moved towards commercial release (Glen Forster, BASF Canada Inc., personal communication). Genotypes containing two resistance genes exhibit increased tolerance to field doses of imazamox, including inadvertent spray overlaps, resulting in reduced crop damage and increased yields (Pozniak et al., 2004b).

Several factors have been identified as potentially affecting tolerance of wheat plants to imazamox including the physiological state of the plant at application, the relative expression of imidazolinone-resistant genes, the rate of enzyme turnover, or other fitness

traits linked to the ALS gene (Hanson et al., 2007). Differences observed between our results and those of Pozniak (2002) and Hanson et al. (2007) suggest that tolerance to imazamox may be greater at the enzyme level than at the whole-plant level in both spring and winter wheat and thus, traits conferring reduced fitness linked to the ALS gene may provide a logical explanation for the observed discrepancies. Further research is needed to determine whether ALS enzyme activity in the absence of imazamox differs between the F_1 and S genotypes used in this study. Future research should also investigate the causes of differential tolerance of wheat plants to imazamox between the enzyme and whole-plant levels in spring wheat genotypes. That similar observations were made between this study conducted with Canadian F_1 genotypes of diverse pedigrees and the near-isogenic American lines used by Hanson et al. (2007) suggests that at least these differences are consistent among studies.

In summary, the results of this study indicate that parental background and market class grouping do not influence the tolerance of spring wheat plants to foliar imazamox application. Although some phenotypic mortality differences among the genotypes tested were observed at 3.9 g a.i. ha^{-1}, phenotypic differences were not observed between F_1 or S genotypes at any other dose. Moreover, shoot biomass accumulation as a result of foliar imazamox application did not differ significantly within each group of F_1 or S genotypes throughout the time course of this experiment. These results were not due to initial biomass accumulation prior to imazamox application. Our results also demonstrate the clear distinguishability of heterozygous hybrids from R and S genotypes in the F_1 generation, and we conclude that IR wheat is a suitable phenotypic marker for identifying hybrids in gene flow studies. Because no differences were observed between parental background or market classes, studies using the IR trait as a phenotypic marker in wheat gene flow studies can reliably apply imazamox at a single dose to discriminate hybrids from homozygous R or S plants.

4.0 Flowering phenology and synchrony between volunteer and cropped spring wheat: implications for gene flow

4.1 Introduction

Hexaploid wheat is one of the most widely-consumed crops in which transgenic cultivars have been proposed for wide-scale introduction. Although transgenic wheat has not been introduced commercially into the agroecosystem, wheat cultivars with transgenic traits are being studied and field tested (Wilson et al., 2003; Finkel, 2008). In Canada, the issue of crop-to-crop and volunteer-to-crop gene flow in wheat is of particular concern given that wheat is Canada's most widely grown crop (nearly 10.5 million hectares in 2006; FAOSTAT, 2008), the majority of the Canadian wheat crop is exported (Stevenson, 2007), and volunteer wheat is very common in Canadian cropping systems. The relative abundance of volunteer wheat in western Canada has increased from the rank of 31 in the 1980s to 12 in the early 2000s (Leeson et al., 2005). During that time, the frequency of fields in which volunteer wheat was observed after in-crop herbicide applications increased from 5.1% in the 1980s to a present day frequency of 10.8% at a mean density of 6 plants m^{-2}. These data are even more remarkable considering that the surveys cannot distinguish volunteer wheat from cropped wheat (the most common crop in this region), indicating that these values are likely a significant underestimation of the true volunteer wheat population.

Transgene movement occurs both spatially (e.g. pollen dispersal) and temporally (via over-wintering of non-harvested grain or volunteers). The tendency of wheat to outcross through pollen flow or be adventitiously present in crops or seed via seed dispersal encompasses the spatial aspect of transgene movement. Volunteer populations facilitate transgene movement in time (Brûlé-Babel et al., 2006). In order to predict the potential

movement of transgenes, studies must be designed to examine the various factors that facilitate movement of the transgene (Lefol et al., 1996; Waines and Hegde, 2003). While many conditions are needed for successful gene flow between crops and volunteers, overlap of flowering time is thought to be a major determinant in the risk of gene flow between two plants (Simard and Légère, 2004). Therefore, temporal isolation of reproduction could be a mechanism that reproductively isolates populations.

Flowering time in spring wheat is largely controlled by a quantitative response to temperature and photoperiod (Marcellos and Single, 1971). Photoperiod may affect the rate of inflorescence development as well as flowering induction *per se* (Dennis, 1984). Genotypes can be classified as either photoperiod sensitive (PS), which require long days to initiate flowering, or photoperiod insensitive (PI), which will flower successfully in long or short day environments (Dyck et al., 2004). Three genes have been reported to control photoperiod sensitivity in wheat including *Ppd-D1* located on the long arm of chromosome 2D, *Ppd-B1* on the short arm of 2B, and *Ppd-A1* on the long arm of 2A (Law et al., 1978; Scarth and Law, 1983). In the case of PI genotypes, the initiation and length of the reproductive phase is controlled primarily by temperature, while both optimal temperature and adequate day length are required in PS genotypes (Horie, 1994). When photoperiod requirements have been met, development of the reproductive phase is primarily a function of temperature, with genotypes developing more rapidly as temperature increases (Halse and Weir, 1970). Spring wheats generally do not have a vernalization requirement (Snape et al., 2001).

Primary factors affecting flowering synchrony between volunteers and crops include emergence timing of volunteers relative to the crop and volunteer density (Simard and Légère, 2004). In some scenarios where a substantial portion of volunteer wheat emerges early in the growing season (De Corby et al., 2007), flowering between the crop and volunteers may be largely asynchronous, and opportunities for transgene movement rare. In other scenarios volunteer wheat emergence can occur in multiple cohorts spanning the entire growing season (Anderson and Nielsen, 1996; Anderson and Soper, 2003), leading to opportunities for significant flowering synchrony and potential transgene movement. In other species such as jewelweed (*Impatiens capensis* Meerb.), flowering phenology

exhibited a plastic response to plant density, resulting in earlier and accelerated flowering periods at high densities (Schmitt et al., 1987). Accelerated flowering phenologies with increasing density have, in fact, been reported for several plant species (Palmblad, 1968).

If transgenic wheat were grown commercially in North America, the large scale production of wheat in this region would result in a considerable portion of transgenic wheat cultivars being grown alongside non-transgenic cultivars with little to no separation distance. Likewise, non-transgenic wheat crops could contain volunteer populations that subsequently acquire a transgenic trait through PMGF. In either case, the only effective barrier to PMGF may be flowering asynchrony between cropped and volunteer wheat. However, no study to-date has examined phenology and synchrony of flowering between volunteer and cropped wheat even though volunteer wheat is sexually compatible with and will emerge and flower within spring wheat crops. Moreover, admixture due to PMGF would be in addition to that caused by seed-mediated gene flow as the volunteer seed is harvested and blended with that of the crop. The objectives of the present study were to: 1) assess the flowering phenology of spring wheat as a function of emergence timing and density; 2) to evaluate (model) the extent of flowering synchrony between cropped and volunteer spring wheat as influenced by volunteer wheat densities and emergence times relative to cropped wheat; 3) to identify a hybridization window (as a function of emergence time) that may facilitate reproductive isolation of spring wheat crops.

4.2 Materials and Methods

4.2.1 Site Description

Field trials were conducted in 2005 and 2006 at Carman (49°29′N, 98°00′W) and Winnipeg (49°68′N, 97°11′W) Manitoba, Canada. Soil at Carman was an Udic Haploboroll, Eigenhof Series (46% sand, 24% silt, 30% clay) averaging 7.5% organic matter and a pH of 5.9, while soil at Winnipeg was a Boralfic Haploborol (20% sand, 26% silt, 55% clay) with 9.1% organic matter and a pH of 6.9. Experiments were located

in different fields within the research farms in both years, with the previous crop being canola and flax (*Linum usitatissium* L.) at Carman and Winnipeg, respectively. Fertilizer was broadcast prior to planting based on soil test recommendations. The experimental area at Carman received a tillage pass in the fall with a field cultivator while the area at Winnipeg was harrowed immediately prior to planting. To ensure low native volunteer wheat populations, experimental areas were chosen that had not been planted to wheat for at least two years. In addition, both experimental areas received a glyphosate application of 900 g a.e. ha^{-1} immediately prior to planting to control emerged weeds.

4.2.2 Experimental Approach

Plots were arranged in a split-plot design with four replications per treatment. The main plot factor was volunteer wheat (cv. CDC Imagine) density and consisted of four target densities: 10, 20, 40 and 80 plants m^{-2}. Time of emergence relative to the crop was the subplot factor and consisted of five volunteer wheat emergence timings. In order to provide a wide range of emergence times for the characterization and modeling of flowering curves, volunteer wheat was planted to emerge at 50 growing degree day (GDD) intervals relative to the wheat crop, providing two treatments targeting emergence at 100 and 50 GDD before the crop (-100 and -50 GDD), a co-emerging treatment (0 GDD), and two treatments following crop emergence (50 and 100 GDD). Subplots were 6.0 m long and 2.0 m wide. Growing degree days were calculated as the cumulative summation of daily average temperature as in Willenborg et al. (2005), where a base temperature of 0.4°C (Addae and Pearson, 1992) and a maximum temperature of 30°C (Cao and Moss, 1989b) were assumed for wheat growth. Planting on a GDD basis provides a more uniformly spaced planting pattern as it accounts for differences in biological activity based on air temperature (Willenborg et al., 2005).

Volunteer and cropped wheat were planted in alternating rows, requiring two passes over each plot. At the third volunteer wheat planting date (0 GDD treatment), the crop (cv. AC Barrie, McCaig et al., 1996) was planted at a target density of 250 plants m^{-2} across all plots. AC Barrie is a commonly grown western Canadian wheat genotype (Canadian Wheat Board, 2008) with a similar maturity requirement as CDC Imagine

(Manitoba Agriculture, Food, and Rural Initiatives, 2008). Planting at all locations was carried out with a low disturbance, double-disc plot drill that established 12 rows on 15.2 cm row-spacing. Post-emergence weed control was achieved at Carman using a tank-mix of clodinafop-propargyl at 56 g a.i. ha^{-1}, thifensulfuron-methyl plus tribenuron-methyl at 7 g a.i. ha^{-1}, bromoxynil plus MCPA ester at 277 g a.i. ha^{-1} with Score adjuvant (1% v/v), and at Winnipeg the same tank-mix was used with the addition of 445 g a.i. ha^{-1} fluroxypyr for volunteer flax control. Preventative disease control was achieved using an application of 124 g a.i. ha^{-1} of propiconazole in all site-years.

Volunteer and cropped wheat emergence were monitored daily on two individual 1 m rows in the high and low density treatments, respectively. Densities of volunteer and cropped wheat were determined by counting the number of plants in two 1 m rows in each plot 3 to 4 wk after crop emergence. When flowering began, flowering was rated daily on ten randomly selected spikes per plot of both volunteer and cropped wheat based on the Zadok's Scale (Zadoks et al., 1974). Because volunteer and cropped wheat were planted in separate rows, volunteers were easily distinguishable from the crop. These data provided a number of estimates for both the volunteers and crop including mean days to 5, 50, and 75% flowering (calculated from Equation 4.3), flowering period duration (calculated as mean days to first and last flower), and flowering overlap between volunteers and crop.

4.2.3 Analytical Approach

Proportional emergence of a population was modelled as a logistic process driven by cumulative GDD:

$$P_t = \left(1 + \exp\left(a\left(-t + b\right)\right)\right)^{-1} \quad (4.1)$$

where P_t is the proportion of seeds emerged after t (GDD) accumulated since the initiation of each volunteer wheat emergence date treatment, a is the estimated rate of emergence (number of emerged seedlings per GDD), and b is the estimated median emergence time (GDD). Final emergence percentage (number of emerged seedlings per unit area divided by that targeted) and median emergence time were then subjected to

analysis of variance using PROC MIXED (Littell et al., 1996), with degrees of freedom calculated by Satterthwaite's approximation method. Means were compared using protected, Bonferroni-corrected multiple comparisons with treatment effects declared significant at $P \leq 0.05$.

Flowering data were initially analyzed with nonlinear regression analysis as a function of time using the NLIN procedure in SAS with iterations derived by the Gauss-Newton algorithm (SAS Inst., 2004). Flowering data obtained from the Zadok's ratings were expressed as a percentage and fit to a four parameter logistic equation:

$$P_f = C + \left(D \cdot \left(1 + \left(x / P_{50}\right)^b\right)^{-1}\right) \tag{4.2}$$

where P_f is the percentage flowering at time x, x is time in Julian days, C is the lower limit of the response curve, $C + D$ is the upper limit of the response curve, b is the slope, and P_{50} is the x value (Julian date) at the midpoint of the curve. Fitting the data to the logistic equation generated a flowering progress curve, which served to facilitate further analysis. Flowering phenology was then characterized by estimating the time required to obtain 5, 50, and 75% flowering for each volunteer wheat relative emergence time and density treatment as determined by the following equation:

$$T_f = P_{50} \cdot \left\{\left[D/\left(y - C\right)\right] - 1\right\}^{\frac{1}{b}} - D_s \tag{4.3}$$

where T_f is time from seeding to 5, 50 or 75% flowering (days), y is percentage flowering, D_s is date of seeding (Julian date), and P_{50}, b, C, D are the nonlinear parameters described above for Equation 4.2.

The distribution of flowering times (Equation 4.2) is a cumulative density function (CDF). In order to assess the degree of overlap among curves, we first calculate the probability density function (PDF) for each population and assess the overlap between the two distributions, which can be mathematically expressed as:

$$f(x) = \frac{dF(x)}{dx} = -\left[b(D)\left(x / P_{50}\right)^b\right] \cdot \left\{\left[1 + \left(x / P_{50}\right)^b\right]^2 x\right\}^{-1} \tag{4.4}$$

where $f(x)$ is the first derivative of the logistic equation (or rate of flowering expressed as percent flowering per day), and b, C, D, P_{50}, and x are the nonlinear parameters described for Equation 4.2. Equation 4.4 provided a good visual representation of both flowering

phenology and overlap for comparison among the treatments. From this, the common area under the various overlapping curves was calculated, providing a direct measure of flowering synchrony. Flowering synchrony for each volunteer wheat relative emergence time and density treatment is the proportion of the area under either PDF when both PDF's overlap. Numerically, this can be calculated by integrating over the minimum value and normalizing by the integral over the entire PDF of either; both integrate to unity as they are probability distributions. Symbolically:

$$F_s = \int_{\infty}^{0} \mathrm{Min}\{f(x), g(x)\}\,\mathrm{d}\,x \tag{4.5}$$

where F_s is the proportion of overlapping area under two curves (flowering synchrony), and $f(x)$ and $g(x)$ are the cumulative flowering proportion (x) at each time interval for volunteer wheat emergence times or densities f and g, respectively. Model calculations were carried out using Mathematica 6.0 (Wolfram Research, 2007). Flowering synchrony values were expressed as percentages and complete synchrony occurs if $F_s = 100$. Because each floret of a wheat spike has an equal opportunity to outcross (i.e. a single floret carries an equal weight to an overall flowering peak), the flowering synchrony calculation is appropriate in that it regards all flowering days as equivalent, independent of the amplitude of flowering (Ollerton and Lack, 1998).

A joint probability distribution was also calculated to provide a prediction of synchronous flowering based on volunteer wheat relative time of emergence and density. The joint distribution was calculated by evaluating the integral of the product of two PDFs:

$$J = \int_{\infty}^{0} f(x) \cdot g(x) \tag{4.6}$$

where J is the joint distribution of the "events" described by the two PDFs, f and g, as described above in Equation 4.5.

Before analyses, all data were examined for normality and homogeneity of variances using the Kolmogorov-Smirnov and Levene's test in SAS, respectively (SAS Inst., 2004). Variances were found to be non-homogeneous between sites and years for all variables. Moreover, extra sum of squares F-tests further confirmed heterogeneity and therefore, analyses were performed within site-year combinations.

Variances were generally normally distributed within site-years and consequently, analyses were conducted on untransformed data in all cases. Heterogeneity of error variances within site-years was modeled using the GROUP option in the REPEATED statement in the mixed-model procedure in SAS (Littell et al., 1996); the WELCH option in the MEANS statement was used when evoking PROC GLM (SAS Inst., 2004). Flowering phenology data and flowering period duration were subjected to analysis of variance within site-years using the mixed model procedure of SAS, with degrees of freedom calculated by Satterthwaite's approximation method. Volunteer wheat density and relative emergence time and their interactions were considered fixed effects, whereas block and its interaction with density were random effects in the model. Because flowering synchrony values and joint probabilities were derived from nonlinear regression analysis conducted across treatments, these values were subjected to analysis of variance within site-years using PROC GLM in SAS (SAS Inst., 2004). The quantitative structure of the treatments necessitated the use of single degree of freedom contrasts using ESTIMATE statements to make comparisons among treatments.

4.3 Results

Median emergence times (time to 50% emergence) and final emergence percentages varied considerably among site-years (Table 4.1). Median emergence times were significantly ($P \leq 0.05$) higher when volunteer wheat emerged before rather than after the wheat crop in 2005. Daily temperatures in mid-May averaged 10°C in 2005 but increased to 15°C by mid-June, likely prolonging the volunteer emergence period for early emerging volunteers (Table 4.1, Fig. 4.1). In contrast, median emergence times were generally greater when volunteers emerged subsequent to the crop at both sites in 2006. Low precipitation in early June of 2006 may have delayed the onset of volunteers emerging after the crop.

TABLE 4.1 Seeding date, accumulated thermal time, observed emergence, median emergence time, and final emergence percentage for volunteer and cropped wheat emergence treatments at Carman and Winnipeg, Canada in 2005 and 2006.

Site-year	Target Emergence	Seeding Date	ATT†	Observed Emergence‡	MET†§	Final Emergence§
	GDD†		GDD†	GDD†	GDD†	%
Carman 2005	-100	May 11	0	-92	135.3 a	93.5
	-50	May 16	28	-64	141.7 a	88.1
	0	May 20	92	0	124.3 b	100.5
	50	May 25	166	75	105.6 c	103.8
	-100	May 30	223	131	117.9 b	97.9
	Crop	May 20	92	0	110.6 c	94.2
Winnipeg 2005	-100	May 12	0	-92	190.3 ab	59.0 ab
	-50	May 16	33	-67	195.9 a	70.8 ab
	0	May 20	100	0	160.7 bc	87.9 ab
	50	May 31	245	145	135.5 c	46.9 b
	-100	-	-	-	-	-
	Crop	May 20	100	0	144.7 c	87.4 a
Carman 2006	-100	May 9	0	-51	112.1 b	88.5
	-50	May 12	43	-28	99.3 c	81.2
	0	May 15	70	0	108.3 bc	86.5
	50	May 18	108	40	105.8 bc	98.9
	100	May 23	164	105	124.7 a	97.4
	Crop	May 15	70	0	109.2 bc	99.4
Winnipeg 2006	-100	May 12	0	-72	110.4 abc	76.6
	-50	May 15	69	-44	101.2 bc	82.0
	0	May 19	120	0	113.3 ab	94.1
	50	May 23	173	50	127.1 a	98.5
	100	May 26	226	97	87.9 c	93.8
	Crop	May 19	120	0	109.1 ab	96.4

†ATT, Accumulated Thermal Time at each volunteer wheat planting date calculated from the first volunteer planting date; GDD, accumulated growing degree days between target planting dates; MET, Median Emergence Time.
‡Observed emergence calculated relative to the crop based on MET. Final emergence within each time of emergence treatment was averaged over densities. Saturated soils precluded planting of the final emergence date at Winnipeg in 2005.
§Within each site-year, means followed by the same lowercase letter were not significantly different ($P \leq 0.05$) as determined by a protected, Bonferroni-corrected multiple comparison procedure.

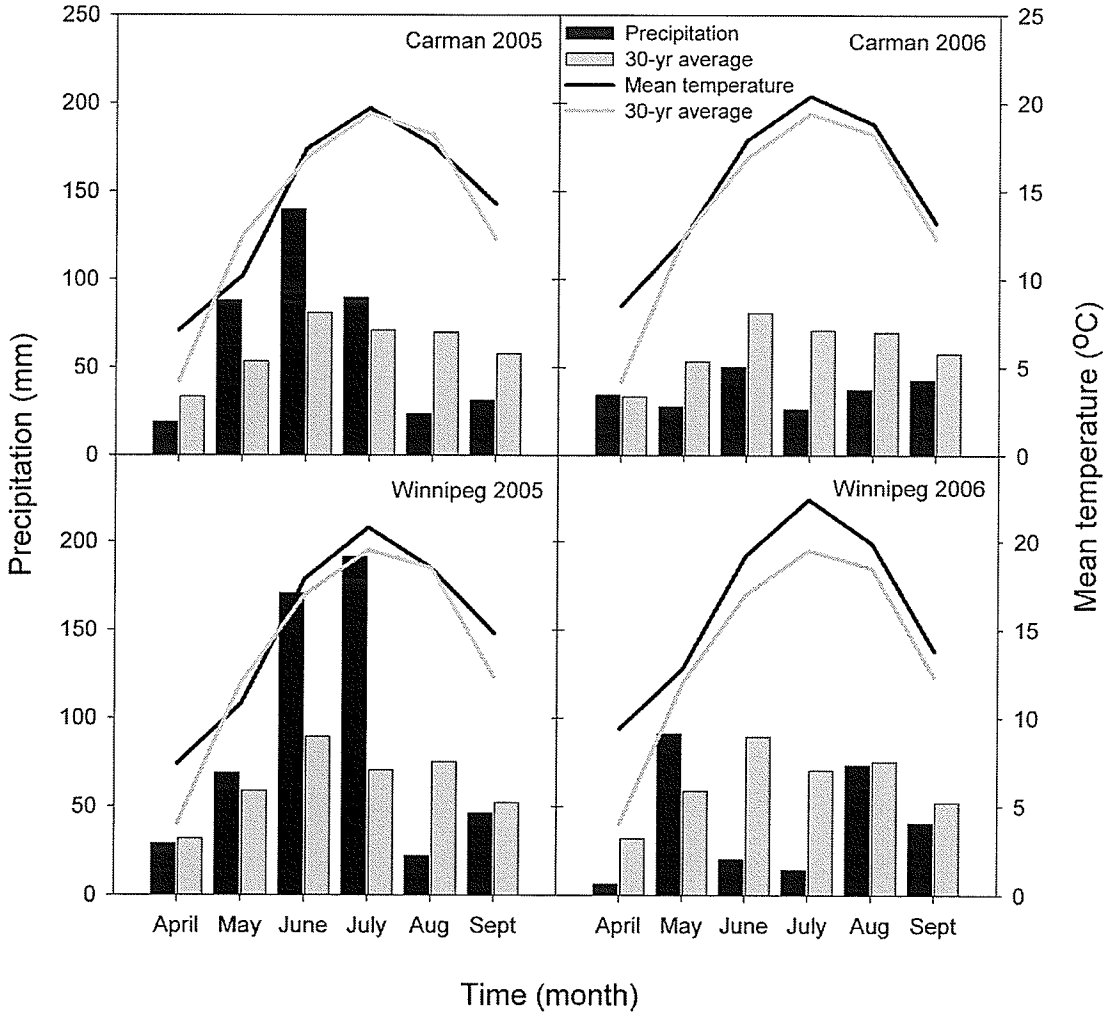

FIGURE 4.1 Mean accumulated precipitation (black bars), 30-year average precipitation (gray bars), mean temperature (black line), and 30-year average temperature (gray line) during the growing season at Carman and Winnipeg, Manitoba, Canada in 2005 and 2006. Thirty-year averages were obtained from Environment Canada (2008) historical weather data.

Relative time of volunteer wheat emergence affected volunteer wheat flowering phenology (Table 4.2). Although analysis of variance indicated significant interactions between treatments for a few variables, their contribution to the total sum of squares was small (1-5%) and thus, they were of little consequence to statistical outcomes. Among site-years, anthesis initiation (time to 5% flowering) varied by as much as 11 d when volunteer emergence occurred 50 GDD after the crop and as little as 6 d when volunteer emergence coincided with the crop (Fig. 4.2). Relative emergence time effects on anthesis initiation were highly significant ($P \leq 0.001$) in all site-years (Tables 4.2 and 4.3), with early emerging volunteers consistently initiating flowering earlier in the growing season than late emerging volunteers (Fig. 4.2). The duration from planting to 5% flowering was consistently reduced as volunteers emerged progressively later in the growing season (Table 4.3). On average, early emerging volunteers (100 and 50 GDD before the crop) took five more days to initiate flowering than late emerging volunteers (50 and 100 GDD after the crop). Generally, volunteer density did not affect crop or volunteer floral initiation timing (Table 4.2).

Progression through the flowering period also was affected by volunteer wheat relative emergence times (Table 4.2). In all site years, time to 50% and 75% flowering was greater ($P \leq 0.05$) when volunteers emerged prior to rather than subsequent to the crop (Table 4.2). Accumulated thermal time to 50% and 75% flowering progressively decreased with increasing time of emergence in all site-years except Carman in 2006 (Table 4.3). Volunteer wheat emerging after the crop required an average (among site-years) of 995 GDD to reach the midpoint of the flowering period as compared to 1020 GDD when emergence preceded the crop. Likewise, a linear decline in time to 50 and 75% flowering was observed with increasing emergence time in all site-years (Table 4.2). Individual emergence cohorts usually had a well-defined flowering peak that was progressively deferred for increasingly delayed emergence times (Fig. 4.2, Table 4.1). Between cohorts in any one site-year, there also was variation in the shapes of the flowering distributions and the timing of peak flowering (Fig. 4.2). For example, there was a much broader spread between peak flowering of the first and final volunteer wheat emergence cohort at Winnipeg (14 d) in 2005 than in 2006 (7 d) (Fig. 4.2). More

TABLE 4.2 Significance (P) of the effect of volunteer wheat time of emergence, density, and their interaction on volunteer wheat time to 5, 50, and 75% flowering as well as crop and volunteer flowering period. Statistically significant ($P \leq 0.05$) P values given by ANOVA are shown in bold.

		P value				
Site-year	Source	Time to 5%	Time to 50%	Time to 75%	Volunteer flowering period	Crop flowering period
Carman 2005	Emergence time (ET)	**0.000**	**0.000**	**0.000**	**0.000**	0.778
	Early vs. Late	**0.000**	**0.000**	**0.000**		
	Linear	**0.000**	**0.000**	**0.000**		
	Density (D)	0.218	0.359	0.259	0.107	0.635
	ET X D	0.163	0.155	0.234	0.191	0.879
Winnipeg 2005	Emergence time (ET)	**0.000**	**0.000**	**0.000**	**0.000**	0.102
	Early vs. Late	**0.000**	**0.000**	**0.000**		
	Linear	**0.000**	**0.000**	**0.000**		
	Density (D)	0.162	0.138	0.165	0.510	0.988
	ET X D	**0.018**	0.733	0.607	**0.030**	0.836
Carman 2006	Emergence time (ET)	**0.000**	**0.000**	**0.000**	0.058	0.351
	Early vs. Late	**0.000**	**0.000**	**0.000**		
	Linear	**0.000**	**0.000**	**0.000**		
	Density (D)	0.126	0.209	0.421	0.633	0.326
	ET X D	0.210	0.570	0.618	0.934	0.994
Winnipeg 2006	Emergence time (ET)	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
	Early vs. Late	**0.000**	**0.000**	**0.000**		
	Linear	**0.000**	**0.000**	**0.000**		
	Density (D)	**0.013**	**0.001**	**0.034**	**0.008**	0.090
	ET X D	0.372	0.070	0.775	0.572	**0.024**

TABLE 4.3 Volunteer wheat relative time of emergence effects on volunteer wheat flowering phenology.

Site year	Observed Emergence (GDD)	(JD) †	Time to 5% (d)‡	(GDD)	Time to 50% (d)‡	(GDD)	Time to 75% (d)‡	(GDD)	Volunteer flowering period (d)‡	Crop flowering period (d)‡
Carman 2005	-92	143	57.9 a	(982)	61.8 a	(1004)	63.1 a	(1032)	6.8 a	4.0
	-60	146	55.5 b	(953)	57.9 b	(1001)	58.8 b	(1023)	6.3 b	4.3
	0	150	53.6 c	(937)	55.7 c	(980)	56.4 c	(980)	4.9 c	4.2
	75	153	52.6 d	(938)	54.5 d	(983)	55.1 d	(982)	4.9 c	4.2
	131	157	49.4 e	(906)	51.7 e	(959)	52.6 e	(977)	6.4 ab	4.3
	Early vs. Late		5.7		6.7		7.0			
Winnipeg 2005	-92	147	59.1 a	(977)	61.6 a	(1049)	62.3 a	(1049)	6.9 a	5.6
	-67	150	56.8 b	(991)	59.2 b	(1061)	60.3 b	(1095)	4.7 b	5.0
	0	152	53.6 c	(950)	57.0 c	(1018)	58.2 c	(1038)	5.1 b	5.4
	145	160	53.0 c	(946)	55.0 d	(981)	55.7 d	(1004)	6.2 a	4.9
	-	-	-		-		-		-	-
	Early vs. Late		4.9		5.6		5.7			
Carman 2006	-51	138	55.6 a	(892)	59.0 a	(965)	60.2 a	(989)	6.1	6.6
	-28	141	54.1 b	(883)	57.6 b	(967)	58.6 b	(979)	6.8	6.1
	0	144	53.4 c	(895)	56.6 c	(966)	57.8 c	(987)	6.4	6.4
	40	146	52.3 d	(902)	55.8 d	(970)	57.0 d	(993)	6.6	5.8
	105	150	49.6 e	(892)	52.6 e	(960)	53.4 e	(982)	5.8	6.1
	Early vs. Late		3.9		4.1		4.1			
Winnipeg 2006	-72	142	55.7 a	(1012)	58.1 a	(1050)	59.0 a	(1067)	5.8 b	4.3 ab
	-44	143	53.1 b	(972)	56.2 b	(1027)	57.3 b	(1049)	4.6 c	4.2 ab
	0	146	51.9 c	(976)	54.5 c	(1020)	55.4 c	(1046)	4.5 c	4.0 b
	50	150	50.0 d	(968)	52.0 d	(1017)	52.3 d	(1040)	4.6 c	4.0 b
	97	151	47.6 e	(940)	50.0 e	(987)	51.0 e	(1010)	7.0 a	4.6 a
	Early vs. Late		5.6		6.1		6.3			

†d, days after seeding; GDD, growing degree days; JD, Julian Date.

‡Means within the same column and site-year followed by the same lowercase letter are not significantly different ($P \leq 0.05$) by single degree of freedom contrasts. Fifth emergence date for Winnipeg in 2005 not available.

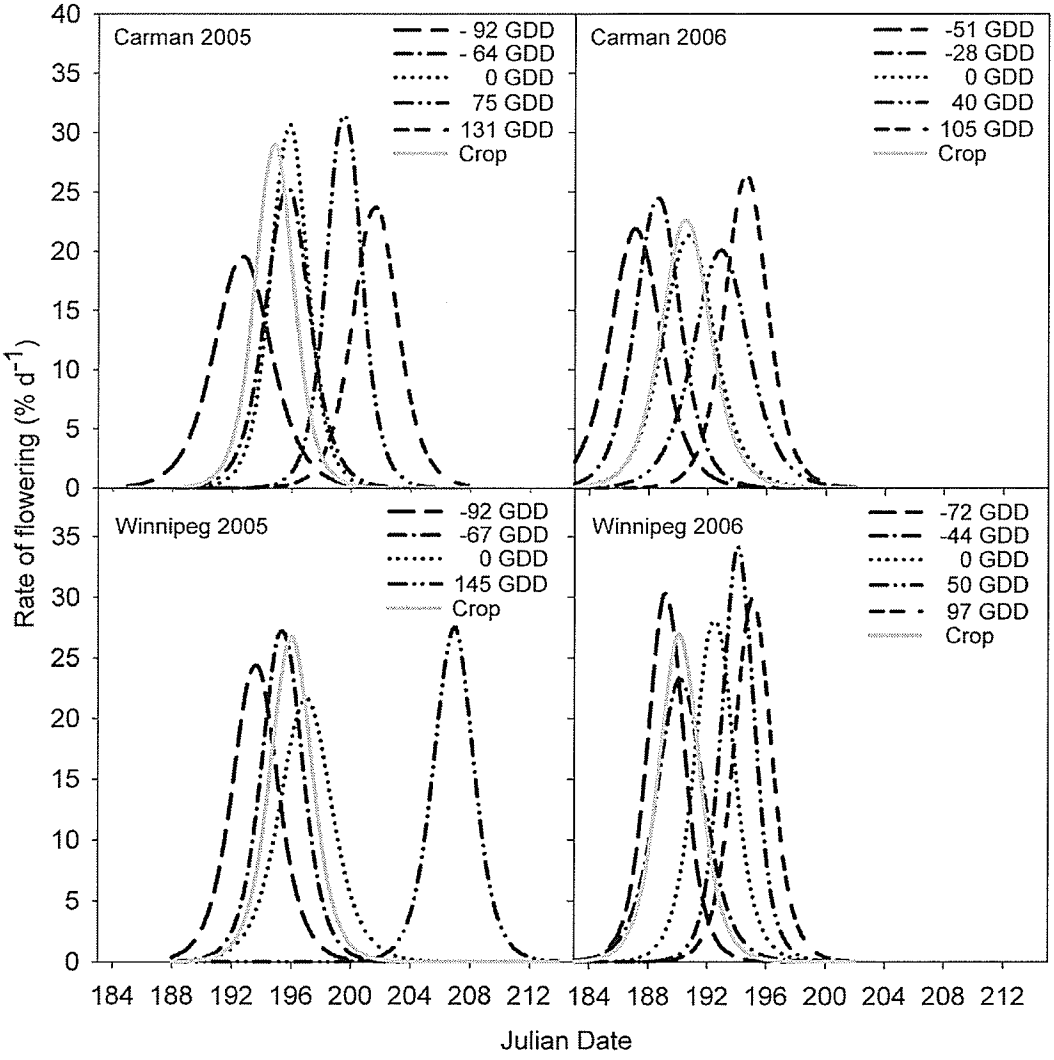

FIGURE 4.2 Rate of volunteer and cropped wheat flowering over time as a function of volunteer wheat time of emergence relative to the crop. Field studies were carried out at Carman and Winnipeg, Manitoba, Canada in 2005 and 2006. Values are the result taking the first derivative (Equation 4.5) of the logistic flowering curve (Equation 4.3) to which raw flowering data were initially fit.

variability in flowering distributions was observed between years than sites, suggesting that environmental conditions influenced the flowering characteristics of wheat more than site-specific conditions. Variability in time to anthesis in wheat between years has been demonstrated in other studies (Pauli et al., 1962; Willey & Holliday, 1971) and because of environmental and genotypic variation, plants within a population rarely exhibit identical flowering phenologies (Elzinga et al., 2007).

Flowering periods between cropped and volunteer wheat overlapped for as many as 7 d and as few as 0.3 d, with overlap ranging from 0% to 82% of total flowering time (Figs. 4.2 and 4.3). Consequently, flowering synchrony between volunteer and cropped wheat was influenced significantly ($P < 0.01$) by volunteer relative emergence time (Fig. 4.3). Flowering synchrony was greatest in all site-years when volunteer emergence coincided with the crop, with mean synchrony values (averaged over sites) of 70% and 84% in 2005 and 2006, respectively. In 2005, volunteers emerging morer than 75 GDD before the crop or 50 GDD after the crop exhibited significantly less flowering synchrony with the crop (range of 0 to 38%). This window shrank to 50 GDD on either side of crop emergence in 2006. Similar results were obtained for joint probabilities, or the probability of synchronous flowering (Fig. 4.3). When volunteer emergence coincided with the crop, there was a greater probability of synchronous flowering than when emergence occurred outside of a 75 GDD window on either side of crop emergence. Averaged across site-years, crop and volunteer wheat emerging at the same time exhibited a one in five chance of synchronous flowering; this declined to a one in ten chance and a one in twenty chance for volunteers emerging greater than 50 GDD before or after the crop, respectively. Density had no effect on either flowering synchrony or joint probability values ($P = 0.95$ to 0.99).

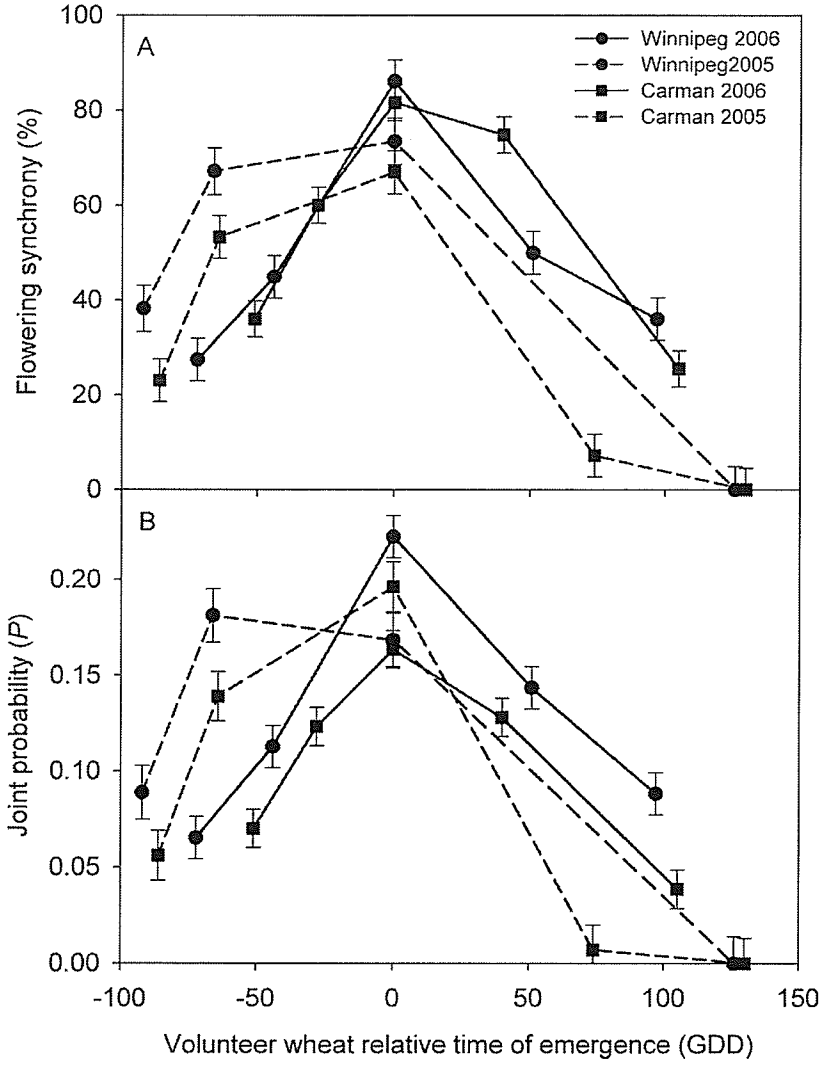

FIGURE 4.3 Relationships between flowering synchrony (A) and the joint probability (B) of flowering synchrony as a function of volunteer wheat time of emergence relative to the crop. Field studies were carried out at Carman and Winnipeg, Manitoba, Canada in 2005 and 2006. Bars indicate ± 1 standard error of the difference between means.

4.4 Discussion

In this study volunteer wheat flowering phenology responded plastically to relative emergence time but not to volunteer wheat density. The timing and shape of flowering curves, as well as peak flowering dates, differed between relative emergence times in most site-years (Table 4.3, Fig. 4.2). Early and late emerging wheat volunteers initiated flowering earlier and later, respectively, and exhibited a longer flowering period than volunteers co-emerging with the crop (Table 4.3). Hence, the window for flowering overlap and gene flow has both a variable width and can be moved in time (Figs. 4.2 and 4.3). These variable responses will have important consequences for patterns of gene flow between transgenic and non-transgenic wheat crops as well as between cropped and volunteer wheat populations. For example, because this window overlapped significantly more with volunteers emerging around the same time as the crop (Fig. 4.3), potential gene flow would be more likely to occur between a transgenic wheat crop and volunteers that emerge at the same time or immediately after the crop, at which point control of the volunteers would be more difficult. Moreover, flowering synchrony and the probability of synchronous flowering also increased substantially when the volunteer emerged at the same time as the crop or within 75 GDD of the crop (Fig. 4.3). Thus, our results suggest a critical period of 150 GDD (75 GDD on either side of crop emergence) where volunteer wheat control in wheat crops is necessary to minimize flowering synchrony and reduce the probability of flowering overlap with the crop. Under field conditions, a 75 GDD interval equates to volunteers having roughly either one more or one less leaf than the crop given a spring wheat phyllochron interval of approximately 80 to 100 GDD (Bauer et al., 1984; Shirtliffe et al., 2000).

Our study identified a mean cumulative thermal time to anthesis of approximately 950 GDD from the time of planting and 834 GDD from emergence (volunteers), which was generally consistent across site-years. These values concur with Bauer et al. (1984) who documented an average of 810 GDD from emergence to anthesis among hard red spring wheat cultivars. Moreover, as volunteer wheat emergence time increased, thermal time requirements for anthesis initiation progressively decreased at all sites except Carman in 2006 (Table 4.3). This is expected for many determinant species as ontogenetic

synchronization results in shortened times to floral initiation as planting is delayed (Horie, 1994). Hunt et al. (1996) also reported a shorter duration from planting to anthesis in wheat cultivars when planting was delayed from April to June. Taken together, these values could help producers and regulators minimize pollination between cropped and volunteer wheat, as well as between adjacent wheat crops, by knowing when flowering will occur and how emergence time affects the pollination period.

Surprisingly, flowering of volunteers was significantly accelerated (flowering period reduced) when emergence coincided with the crop compared to when volunteers emerged more than 72 GDD before or 100 GDD after the crop (Table 4.3). Differences in volunteer flowering period ranged from as little as 1.4 d at Carman in 2005 to as much as 2.5 d at Winnipeg in 2006. This observation may reflect light quality sensitivity in the volunteer genotype and the effects of density. Low red:far-red ratios characteristic of high plant densities are known to accelerate flowering (Ballaré, 1999; Smith, 2000; Faigón-Soverna et al., 2006). Nevertheless, these seemingly small differences were statistically significant ($P \leq 0.05$) and represented a 39% and 56% reduction in flowering period duration in those site-years, respectively. In contrast, duration of crop flowering period was affected by volunteer relative emergence time in only one site-year (Table 4.2). Flowering periods of cropped and volunteer wheat were largely unaffected by volunteer wheat densities (Table 4.2). Mean volunteer and cropped wheat flowering durations were 4.8 and 4.1 d in our study, respectively, which agrees well with the 3.3 d average for Canada western hard red spring varieties reported by Hucl (1996).

Emergence timing of spring wheat volunteers under field conditions can vary. De Corby et al. (2007) reported median emergence times under Canadian conditions occurred in mid to late April, ranging between 156 and 235 GDD with emergence occurring in a single cohort. In this case, delayed planting of wheat crops could successfully be used to control volunteer wheat prior to seeding. However, both Anderson and Nielsen (1996) and Harker et al. (2005) have observed sporadic volunteer wheat emergence periodically throughout the growing season in multiple cohorts; delayed planting of wheat crops in this case will not suffice and adequate control of volunteer plants in-crop would be difficult. Even if transgenic wheat possesses resistance

to nonselective herbicides (HR), further applications of the specific herbicide in future generations will continue to select for resistant volunteers where hybridization between volunteers and the HR crop has previously occurred, promoting potentially rapid spread of the transgene. Selection pressure and gene flow can profoundly influence the spread of a HR transgene between wheat populations (Brûlé-Babel et al., 2006). Moreover, high densities of wheat volunteers whose emergence coincides with wheat crops could be auspicious for hybridization given the high degree of flowering overlap observed in our study (Fig. 4.3). While it is conceivable that the chances of hybridization with the crop would be higher when there are more volunteers present, our results suggest that volunteer density does not impact flowering phenology or synchrony and therefore, may not affect hybridization at densities generally observed under field conditions (Table 4.2). In western Canada, average densities of volunteer wheat populations is approximately 6 wheat plants m^{-2} following postemergence herbicide applications, but may range up to fifty times this value; Leeson et al. (2005) reported densities as high as 281 plants m^{-2}.

In summary, volunteer wheat density did not influence flowering phenology and synchrony of either cropped or volunteer wheat at densities commonly experienced in the field. Volunteers co-emerging with the crop exhibited an accelerated flowering period and substantial levels of synchrony with the crop and thus, appear poised to contribute gene flow at levels greater than volunteers emerging outside of a 75 GDD critical period on either side of crop emergence. Our novel approach to studying volunteer and crop flowering synchrony should provide for an improved ability to relate flowering phenology and synchrony observations to other variables such as pollen flow, viability, and gene flow. However, further research is necessary to determine the relationship between flowering synchrony and gene flow in wheat and to determine if flowering asynchrony presents an exploitable barrier to gene flow. The results of this study will provide a basis for the development and parameterization of mechanistic models simulating novel trait and transgene movement in wheat crops.

5.0 Emergence timing facilitates temporal isolation of pollen-mediated gene flow between volunteer and cropped spring wheat

5.1 Introduction

The use of genetic engineering for crop improvement and the accompanying cultivation of transgenic plants is a topic of considerable global debate. The potential for gene flow between transgenic and non-transgenic crops remains a concern that must be addressed satisfactorily prior to their introduction. Inadvertent movement of plant traits can occur through the escape of viable seeds into commerce or through the sexual transfer of traits via pollen (Waines and Hegde, 2003; Demeke et al., 2006). Often referred to as pollen-mediated gene flow (PMGF), movement of traits via pollen may be evaluated by observing outcrossing from pollen source to receptor plants (Halsey et al., 2005). Pollen-mediated gene flow is of concern because of the profound influence it may have on population genetic structure (Slatkin, 1987). However, populations that can be reproductively isolated, either spatially or temporally, are unlikely to be affected by PMGF (Brûlé-Babel et al., 2006).

Wheat is a self-pollinating, anemophilous, crop that produces abundant pollen for fertilization. An individual anther of wheat produces between 1750 and 3900 pollen grains (Joppa et al., 1968; De Vries, 1974a), of which as much as 80% may be shed beyond the glumes (D'Souza, 1970). Adverse conditions can increase the quantity of external pollen shed and it is estimated that only 30% of pollen shed occurs within the floret, with the rest available for PMGF (Leighty and Sando, 1924). Moreover, low moisture and high stress conditions also promote chasmogamous flowering, thus

increasing the potential for PMGF (Waines and Hegde, 2003). Despite 90% of wheat pollen being dispersed by gravity downward from the spike and falling within 6 m of the originating plant (Jensen, 1968), wheat pollen grains have been detected as far as 1000 m from the pollen source (Virmani and Edwards, 1983). Therefore, the evidence to-date suggests that pollen densities outside the floret are great enough to be conducive to outcrossing, creating the risk of low levels of PMGF with volunteer populations or neighboring wheat crops.

Given that transgenic wheat is currently being field tested (Finkel, 2008), the issue of PMGF in wheat requires prompt attention. A number of recent studies have investigated natural PMGF in wheat, where outcrossing was measured between phenotypically or genetically distinct pollen source and receptor populations. Martin (1990) reported that outcrossing frequencies in winter wheat varied from 0.1 to 5.6% among individual genotypes within any given year. Hucl (1996) measured outcrossing among 10 Canadian spring wheat cultivars and found that outcrossing varied between 0 and 6.7%, with a mean of 0.88%. Using a blue aleurone spring wheat cultivar, Hucl and Matus-Cádiz (2001) reported that the frequency of PMGF from a 25 m^2 source plot declined from 3.8% adjacent to the source plot to 0.2% 30 m from the source plot. A more extensive study, which measured outcrossing from a 0.25 ha pollen source at distances of up to 150 m, found the frequency of PMGF declined from 0.44% 1 m from the source to negligible levels beyond 60 m (Matus-Cádiz et al., 2004). However, PMGF was confirmed at 300 m from the source by Matus-Cádiz et al. (2004), and a subsequent study has detected PMGF as far as 2.75 km from a 33 ha pollen source (Matus-Cádiz et al., 2007). Similar declines in PMGF with increasing distance from a pollen source were reported in the Pacific Northwest region of the USA (Hanson et al., 2005b) and Australia (Gatford et al., 2006).

Although the above studies demonstrate that PMGF occurs naturally under field conditions and at considerable distances, distance can act as a barrier to PMGF. However, distance alone is only one possible barrier to PMGF and should transgenic wheat be grown commercially, many transgenic wheat crops will border non-transgenic wheat crops. Wheat is the most widely grown crop in Canada, with more than 38% of the arable land utilized for crop production devoted to its cultivation annually (Statistics Canada,

2006). Moreover, volunteer wheat plants growing within a non-transgenic wheat crop may also acquire and spread transgenes through PMGF. Volunteer wheat is the 12th most abundant weed on the Canadian Prairies, and is present on about 11% of fields at densities as high as 280 plants m^{-2} (Leeson et al., 2005). In such cases, temporal isolation of the synchronously reproducing populations may represent the only barrier to PMGF.

The concepts of hybridization and reproductive isolation are antithetic and consequently, the probability of hybridization between two populations or plants is directly dependent upon the extent to which they are reproductively isolated (Richards, 2005). Temporal isolation, the deliberate disruption of flowering synchrony, a key prerequisite for hybridization, is usually achieved by displacing sowing dates with the goal of enforcing reproductive isolation (Halsey et al., 2005). Temporal isolation is a well-established mechanism for genetic isolation of populations (Richards, 2005), but has not been as extensively quantified as separation distance. In fact, few reports exist of two populations being at least partially isolated by flowering time in the agroecosystem, and even fewer have quantified the relative importance of flowering time in facilitating temporal reproductive isolation. Halsey et al. (2005) reported that increasing temporal separation reduced the distance required to achieve genetic isolation (outcrossing <0.01%) in maize (*Zea mays* L.) from 500 m when source and receptors flowered synchronously to 62 m or less when 2 wk of temporal isolation was attained. Also in maize, Della-Porta et al. (2008) found little to no reduction in outcrossing with only 3 d difference in flowering time between source and recipient, but a difference in flowering of 6 d reduced outcrossing by nearly 50%.

In order for transgenic wheat to be integrated into commercial agriculture, it is essential to understand the potential for transgene movement within the environment where the crop will be grown (Waines and Hegde, 2003). Volunteer wheat is an abundant weed in western Canada that can emerge either in a single cohort early in the growing season (De Corby et al., 2007) or in multiple cohorts spanning the length of the growing season (Anderson and Soper, 2003; Harker et al., 2005). Chapter 4 demonstrated that emergence timing of volunteers was fundamental to flowering synchrony between cropped and volunteer wheat populations (Willenborg et al., 200x), and it is highly probable that the

same effects will be evident in PMGF. To this end, the objectives of the research reported in this chapter were to: 1) determine whether variable emergence timing of volunteer spring wheat could temporally isolate volunteer and cropped wheat populations, thus minimizing PMGF; 2) to identify a hybridization window in spring wheat crops based on the emergence timing of volunteers and the resulting temporal isolation; 3) to ascertain whether a relationship exists between flowering synchrony and PMGF as a function of emergence timing and density of volunteer wheat plants. Gene flow, as referred to hereafter, is defined as "the incorporation of genes into the gene pool of one population from one or more other populations" (Futuyma, 1998). Thus, PMGF requires an initial pollination event that results in the production of viable seed containing the trait of interest.

5.2 Materials and Methods

5.2.1 Site Description

Field studies were conducted from 2005 to 2006 near Carman (49°29′N, 98°00′W) and Winnipeg (49°68′N, 97°11′W), Manitoba, Canada. Trials in Carman were placed on an Udic Haploboroll (sandy loam) with 7.5% organic matter and a pH of 5.9. They soil type at Winnipeg was a Boralfic Haploborol (clay loam) averaging 9.1% organic matter with a pH of 6.9. An experimental area was chosen at each location that had not been planted to wheat for at least two years. Both sites received a glyphosate application at 900 g a.e. ha^{-1} just prior to sowing to control emerged weeds, including any volunteer wheat plants that were present. Experiments were conducted at least 300 m from an adjacent wheat field to further isolate the experimental area from competing pollen.

5.2.2 Experimental Design and Procedures

Plots were established and maintained using recommended agronomic practices for spring wheat production in western Canada. Liquid fertilizer (50-0-0-20) was applied as a dribble band at Winnipeg while at Carman, granular fertilizer (46-0-0-0) was broadcast

and incorporated prior to planting; specific nutrients and rates were based on soil test recommendations (target yield 4700 kg ha^{-1}) for each site. Planting rates were adjusted for thousand kernel weight and germination test results. Both volunteer and cropped spring wheat populations were established using a low-disturbance, double-disc plot drill with 15.2 cm row spacing. Weed and disease control was achieved using recommended, commonly-used chemicals standard for spring wheat production.

In order to model the influence of various volunteer emergence timings and densities on PMGF, volunteer wheat populations were established. Factorial combinations of volunteer wheat density and time of emergence (relative to the crop) treatments were arranged in a split-plot experimental design with four replications. Isolation distances of 10 m between each main plot, 10 m between reps, and 3 m between subplots were implemented to minimize plot-to-plot outcrossing (Fig. 5.1). Main plot treatments (6 x 22 m) were comprised of volunteer wheat densities of 0, 20, 40, and 80 plants m^{-2}. Five volunteer wheat emergence timings at 50 growing degree day (GDD) intervals relative to the crop comprised subplots (6 x 2 m). Growing degree days were calculated as the cumulative summation of daily average temperature as in Willenborg et al. (2005), where a base temperature of 0.4°C (Addae and Pearson, 1992) and a maximum temperature of 30°C (Cao and Moss, 1989) are assumed for wheat growth. Because spring wheat can flower for as few as 3 d (Hucl, 1996) to as many as 9 d (Matus-Cádiz et al., 2004) it was necessary to have emergence times adequately spaced to fully envelope flowering of the spring wheat crop (receptor) with volunteer wheat pollen (source). Thus, the study employed a planting regime consisting of two treatments targeting emergence 100 and 50 GDD before the crop (-100 and -50 GDD), one at crop emergence (0 GDD), and two 50 and 100 GDD following crop emergence. To facilitate visual identification of each population, a paired-pollinator row arrangement was utilized, resulting in pollinator (volunteer) and source (crop) plants being sown in alternating rows. A receptor population of cv. AC Barrie (McCaig et al., 1996) spring wheat was established at a target density of 250 plants m^{-2} at the third volunteer wheat planting (0 GDD treatment). AC Barrie was chosen because it is a widely grown western Canadian spring wheat genotype (Canadian Wheat Board, 2008). CDC Imagine, an imazamox-resistant (IR)

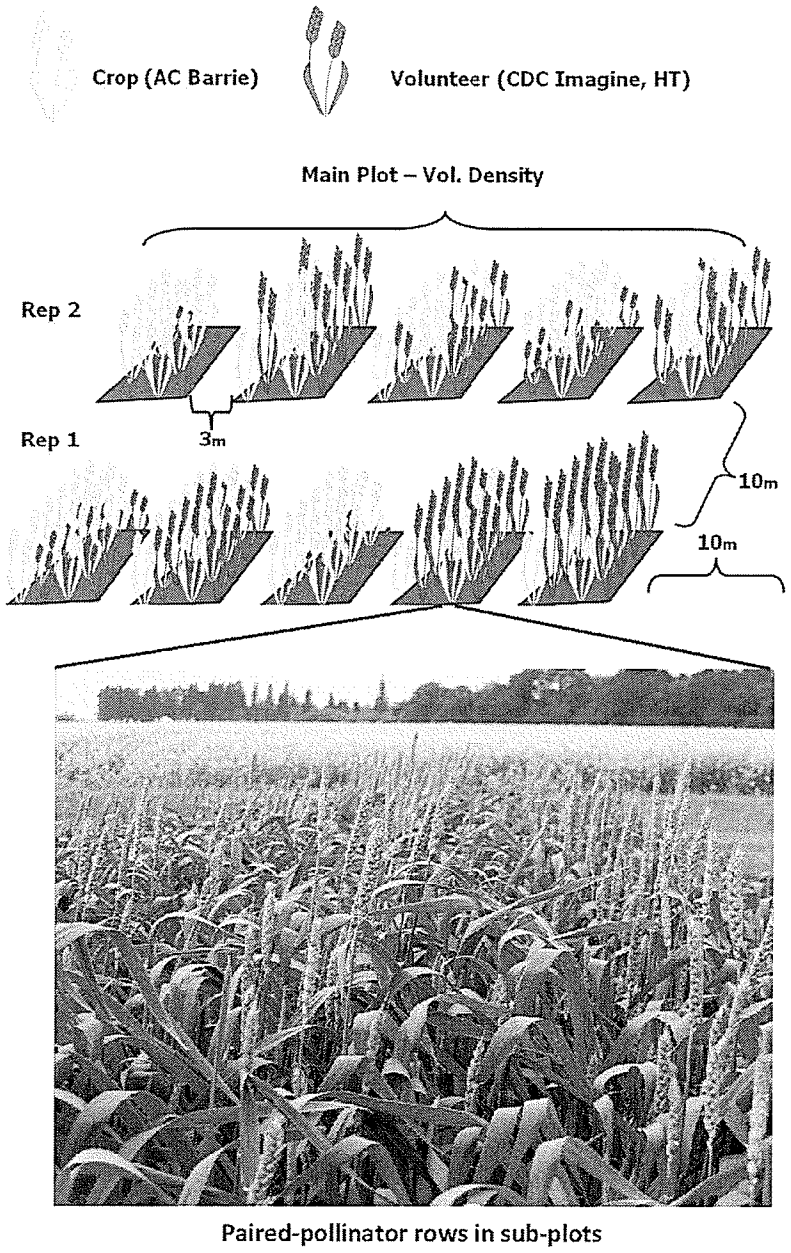

FIGURE 5.1 Illustration of the experimental design for investigating pollen-mediated gene flow from volunteer to cropped wheat populations. Volunteer (green plants) density comprised main plots while subplots consisted of various volunteer emergence timings relative to the crop (yellow plants). Planting pattern in each plot was that of a paired-pollinator row, consisting of alternating rows of volunteer and cropped wheat. Isolation distances of 3, 10, and 10 m were implemented for subplots, main plots, and replicates, respectively.

spring wheat genotype was used to establish the volunteer (pollinator) population.

Volunteer and cropped spring wheat emergence were counted daily in two individual 1 m row lengths per plot in low and high density treatments, respectively. Plant densities were determined by recording the number of emerged plants in two 1 m rows in each plot at the 2- to 3-leaf stage of the crop. A subsample of spikes from each plot was harvested by hand from two 1 m rows of crop, threshed immediately using a plot harvester, placed in individually labeled cloth bags, and transported to a central processing facility where they were dried to a uniform moisture. At the same time, a second subsample of cropped wheat spikes was cut by hand from another 1 m row in each plot, the total number of spikes counted, and five spikes from each sample hand-threshed to estimate the number of seeds spike^{-1}. Grain samples were cleaned using a dockage tester and hand-cleaned of any remaining foreign material. Individual grain samples were then weighed (to determine grain yield) and the total weight of each sample divided by the average weight of 1000 seeds (TKW) to estimate the total number of seeds in each sample. Thousand kernel weight was calculated by determining the weight of 500 kernels and multiplying by two. Air temperature, relative humidity, and precipitation were measured throughout the flowering period by a weather station located within 200 m of each experimental area. Average air temperature and relative humidity were recorded approximately 1 m above the soil surface at 1 h intervals during flowering. De Vries (1972) demonstrated that the diurnal period during which pollen shed is greatest in wheat is between 0600 to 1800 h. Therefore, air temperature and relative humidity values were averaged during this period to derive mean daily air temperature and relative humidity estimates.

5.2.3 Sample Screening

Pollen-mediated gene flow was analyzed by testing for imazamox herbicide resistance in the crop seedling progeny from each field trial plot. Approximately 2000 seedlings per plot (harvested the year prior) from each grain sample (one per plot), along with control plots for each of the resistant (CDC Imagine) and susceptible (AC Barrie) genotypes were screened for imazamox resistance. A foliar application of imazamox (BASF Canada Inc., Toronto, ON) was applied at a rate of 20 g a.i. ha^{-1} to wheat seedlings at the 2- to 3-

leaf stage using a plot sprayer calibrated to deliver 56 L ha^{-1} spray volume at 275 kPa, with Merge® surfactant added at 0.5% v/v to the spray solution. All surviving wheat seedlings were counted approximately 16 d after herbicide application based on surviving plants exhibiting an injured phenotype, which included coleoptilar tillering, minor chlorosis, deep greening of developed leaves, and stunting (Pozniak et al., 2004a).

All surviving plants (where logistically possible) were then grown to maturity, hand-harvested, and transported back to the greenhouse for a second confirmatory screening. Sixteen wheat grains from each putative hybrid plant were germinated in 27 x 54 cm plastic trays, with two seeds sown in each of 48 individual cells. Trays were placed in a growth room (16-h photoperiod, 21/15°C day/night temperature, radiance of 480 μmol m^{-2} s^{-1}) and sprayed with imazamox at 12 g a.i ha^{-1} using a cabinet sprayer calibrated to deliver 117 L ha^{-1} of spray solution at 310 kPa in a single pass over the foliage, with Merge® added at 0.5% (v/v) to the spray solution. In Chapter 3 (Willenborg et al., 2008) we demonstrated that the optimal dosage of imazamox for growth room discrimination of R, S, and heterozygous intermediate (I) plants is 12 g a.i. ha^{-1}. Hybridization (PMGF) was confirmed by classifying segregation among $F_{1:2}$ seedlings as segregating (3 alive: 1 dead ratio) or nonsegregating (either all alive or all dead) for the single gene, semi-dominant imazamox-resistant trait (Pozniak and Hucl, 2004). A total of 18, 256 $F_{1:2}$ seedlings were screened from the 1, 241 putative hybrids harvested across the four location-year combinations.

5.2.4 Statistical Analysis

The UNIVARIATE procedure of SAS (SAS Institute, 2004) was used to assess normality and homogeneity of residuals prior to all analyses. Data conformed to the assumptions of ANOVA and therefore, crop yield and yield component data were analyzed using the mixed-model procedure of SAS (Littell et al., 1996). Volunteer wheat density, emergence time, and their interaction represented fixed effects, whereas blocks, locations, years, and their interactions with fixed effects were considered random.

Pollen-mediated gene flow was expressed as a percentage and calculated as follows:

$$\text{PMGF (\%)} = (\text{number of confirmed heterozygotes in a sample/ total number of seeds in a sample}) \times 100 \quad (5.1)$$

To model PMGF as a function of density and emergence timing, an estimation of median emergence timing relative to the crop is required. This information was derived from fitting the following logistic equation to each experimental unit (Willenborg et al., 2005):

$$P_t = (1 + \exp(s(-t + m)))^{-1} \quad (5.2)$$

where P_t is the proportion of seedlings emerged at time t, t is accumulated thermal time (GDD) from the initiation of each volunteer wheat emergence treatment, s is the estimated rate of emergence (number of seeds emerged per GDD), and m is the estimated median emergence time (GDD) for each population in each experimental unit.

Following this, it is possible to both identify the hybridization window in spring wheat and examine the reproductive isolation based on emergence timing by fitting a model consisting of a hyperbolic equation (Cousens, 1985) linked to a Gaussian function:

$$Y_{GF} = \frac{iD_v}{1 + iD_v / a_{\max}} e^{-\left(\frac{E_t - c}{f}\right)^2} \quad (5.3)$$

where Y_{GF} is the frequency of pollen-mediated gene flow (expressed as a percentage), D_v is volunteer wheat density (plants m^{-2}), E_t is the actual median emergence time of volunteer wheat relative to the crop in GDD (calculated with Equation 5.2), and $a_{\max}$, i, c, f are fitted model parameters. Parameter $a_{\max}$ describes the amplitude of the curve or the maximum estimated gene flow as $D_v \rightarrow \infty$, i describes the rate at which Y_{GF} declines as $D_v \rightarrow 0$ and $E_t \rightarrow c$, c is the shift of the curve peak from 0 on the x-axis, and f is the deviance of the curve from the curve peak (spread of the curve). Both equations 5.2 and 5.3 were fit to the data using nonlinear regression (PROC NLIN, SAS Inst., 2004), with iterations derived by the Marquardt procedure.

Unique plant densities within each experimental unit precluded an estimation of pure error and ultimately, lack of fit tests could not be performed. Therefore, several statistics, including a model F statistic, a *pseudo* R^2 (Schabenberger and Pierce, 2002), mean square prediction error (MSPE), and root of MSPE (rMSPE) expressed as a percentage of the

observed mean (Thiel, 1966) were used to evaluate goodness-of-fit of each regression model. Mean square prediction error was calculated as:

$$MSPE = \frac{1}{n}\sum_{i,j=1}^{n}\left(P_{ij} - A_{ij}\right)^2 \tag{5.4}$$

where P_{ij} is the predicted value at volunteer wheat emergence timing i and density j, and A_{ij} is the observed value at emergence timing i and density j. Root MSPE was also calculated (MSPE divided by the observations mean) to express MSPE in the same units as observed and predicted variables (Fathi Nasri et al., 2006).The MSPE was then decomposed into mean bias or error in central tendency (ECT), slope bias or error due to regression (ER), and random error due to disturbance (ED). Calculation of these three components, with each expressed as a percentage of the MSPE, is as follows (Bibby and Toutenburg, 1977):

$$\text{ECT} = \left(\overline{P} - \overline{A}\right)^2 \tag{5.5}$$

$$\text{ER} = \left(S_P - rS_A\right)^2 \tag{5.6}$$

$$\text{ED} = \left(1 - r^2\right)\cdot S^2{}_A \tag{5.7}$$

where $\bar{P}$ and $\bar{A}$ are the averaged predicted and observed values, respectively; S_p and S_A are the standard deviations of the predicted and observed values, respectively; and r is the correlation coefficient between predicted and observed values. Error in central tendency measures the deviation of average predicted values from average observed values, whereas error due to regression indicates deviation of the least squares regression coefficient from one (expected value if predictions were completely accurate). Error due to disturbance represents the variation in observed values that remains unexplained once the mean and regression biases have been removed (Fathi Nasri et al., 2006). Data sets for the regressed variables in Equation 5.3 were then systematically compared for differences in parameter estimates using a stepwise process based on the extra sum of squares principle for nonlinear regression described by Ratkowsky (1983).

Correlations were carried out to examine the relationships between yield components, flowering synchrony and joint probability distribution data from Chapter 4, and PMGF. Pearson correlations were used when residuals were normally distributed, whereas non-

parametric Spearman Rank correlations were employed when residuals were non-normal and could not be transformed. Where significant correlations were found between PMGF and other variables, linear regression (PROC REG, SAS Inst., 2004) was used to further examine the relationship between regressor and response (PMGF) variables.

5.3 Results

Daily average temperatures during the pollen shed period ranged from 13.8 °C to 30.5°C, and average relative humidity was 39.0 to 95.9% (Fig. 5.2). Although there were no remarkable differences in these two parameters between locations or years, relative humidity tended to be higher at both locations in 2005 compared to 2006, owing to greatly increased precipitation at both sites in 2005 (data not shown). Individual volunteer wheat emergence cohorts flowered for 7 to 9 d, while the wheat crop flowered for 7 d irrespective of location or year (data not shown). However, the cumulative volunteer flowering period (totality of all cohorts) was 9 d shorter at both sites in 2006 as a result of the higher average temperatures (Fig. 5.2). The greatest flowering synchrony in all years and locations was obtained when volunteer emergence coincided with the crop, but there was always at least one day of flowering overlap between volunteer and cropped wheat populations when volunteers emerged within 150 GDD of the wheat crop (Chapter 4; Willenborg et al., 200x).

A total of ca. 545,407 wheat seedlings were screened for hybridization among locations and years. Approximately 34% (105) of the 309 samples examined over two locations and two growing seasons were confirmed as having IR hybrids present (Table 5.1). Segregation ratios of suspected hybrids consistently fit the expected 3:1 phenotypic ratio for a single, semi-dominant gene in the $F_{1:2}$ generation at both sites in 2005 and 2006 (Appendix 1). The maximum PMGF frequency in samples where IR hybrids were identified was 0.514%, while the mean frequency of PMGF in those samples (averaged across the experiment) was 0.218%. By contrast, accounting for the 66% of plots where

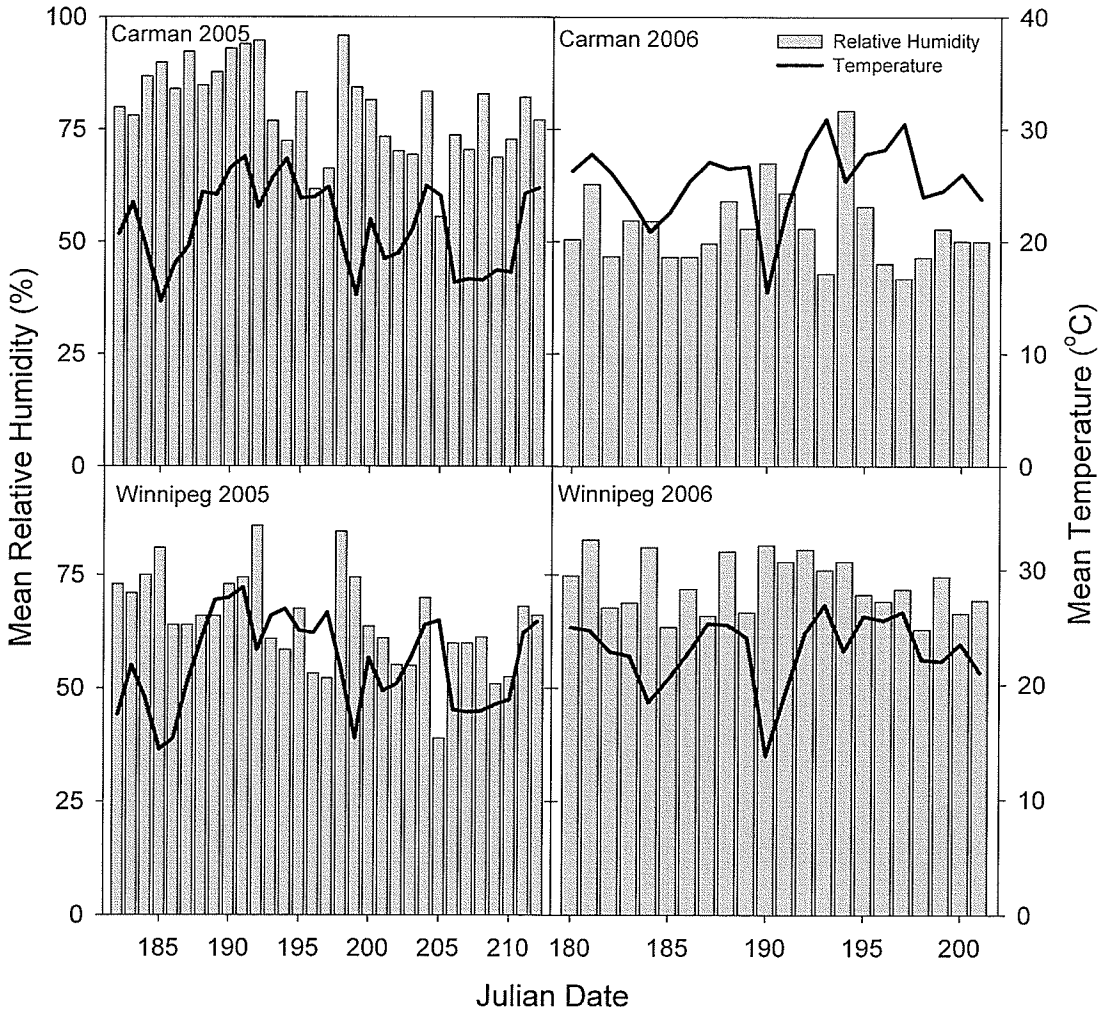

FIGURE 5.2 Mean daily air temperature (black lines) and relative humidity (gray bars) during the pollination period at Carman and Winnipeg, Manitoba, Canada, in 2005 and 2006. Values were obtained from weather stations located within 200 m of each site and represent the average of values recorded at one hour intervals between 0600 and 1800 h.

TABLE 5.1 Descriptive statistics of pollen-mediated gene flow in wheat samples of various emergence treatments in 2005-2006 spring wheat gene flow experiments.

Site	Year	Emergence treatment†	Emergence date	Flowering date	Samples		Pollen-mediated gene flow			
					Analyzed‡	With outcrossing§	Min.	Max.	Mean	Standard error
		GDD					%			
Carman	2005	-100	May 22	July 8	14	1	0.098	0.098	0.098	-
		-50	May 23	July 11	16	3	0.120	0.199	0.161	0.023
		0	May 28	July 13	16	9	0.089	0.388	0.185	0.035
		50	June 1	July 17	15	3	0.035	0.311	0.158	0.035
		100	June 5	July 18	15	3	0.072	0.195	0.126	0.036
Winnipeg	2005¶	-100	May 28	July 10	14	3	0.046	0.089	0.067	0.013
		-50	May 30	July 12	14	3	0.095	0.468	0.245	0.113
		0	June 1	July 13	15	6	0.054	0.354	0.153	0.044
		50	June 14	July 20	16	2	0.148	0.196	0.148	0.048
		-	-	-	14	0	-	-	-	-
Carman	2006	-100	May 18	July 3	16	7	0.029	0.171	0.096	0.021
		-50	May 20	July 5	16	7	0.057	0.200	0.117	0.022
		0	May 23	July 7	16	13	0.089	0.514	0.184	0.038
		50	May 25	July 9	16	11	0.014	0.240	0.091	0.020
		100	May 29	July 12	16	6	0.019	0.160	0.097	0.022
Winnipeg	2006	-100	May 22	July 7	16	2	0.058	0.058	0.058	-
		-50	May 23	July 7	16	8	0.058	0.185	0.112	0.015
		0	May 26	July 10	16	10	0.081	0.296	0.157	0.023
		50	May 30	July 12	16	5	0.068	0.232	0.163	0.029
		100	May 31	July 13	16	3	0.058	0.162	0.108	0.030

† Emergence treatments are target emergence timing relative to the crop in growing degree days (GDD). See Materials and Methods for more details.

‡Number of samples analyzed out of a possible 16 samples per emergence time treatment per location. Some samples had no grain due to adverse weather conditions.
§Number of samples with at least one confirmed hybrid plant.
¶Volunteers in the 100 GGD emergence time treatment could not be planted at Winnipeg in 2005 due to unseasonably high precipitation. However, these plots were harvested to determine the frequency of plot-to-plot PMGF rate, which was found to be undetectable.

PMGF was not detected, the average frequency of hybridization ranged from a low of 0.023% at Winnipeg in 2005 to a high of 0.069% at Carman in 2006, and summed over years was nearly two-fold higher at Carman than at Winnipeg.

The multiplicative model (Equation 5.3) provided a statistically significant ($P < 0.001$) fit to the PMGF data set, with 71% of the total variance in spring wheat PMGF explained by the model (R^2) and a relatively small rMSPE (Table 5.2). Plots of residuals indicated that errors generally conformed to the underlying regression assumptions (data not shown). Decomposition of the MSPE indicated that deviation between predicted and observed values (ECT) and regression bias (ER) were both extraordinarily small (<1% of MSPE). Large values of these statistics would suggest inadequacies of the model to predict the variable in question (Fathi Nasri et al, 2006). In addition, all four parameters were well-estimated as indicted by their small standard errors (< 30% of estimate) . Standard errors that are less than half of the numerical value of the parameter estimate indicate good estimation (Koutsoyiannis, 1973). Moreover, systematic statistical testing using the ESS principle revealed that parameter estimates did not differ significantly across years or locations and thus, a single, combined model in which data were pooled across years, locations, and blocks was fit to the data (Table 5.3). The multiplicative model (Equation 5.3) developed here provided a good description of all PMGF data across years and locations.

Although no relevant, statistically significant treatments effects were detected for spring wheat crop grain yield or yield components data (data not shown), a substantial interactive effect of both volunteer wheat density and emergence timing on PMGF frequencies was observed (Fig. 5.3). Relatively low frequencies of PMGF were observed regardless of volunteer wheat emergence timing or density. The maximum estimated PMGF (a_{max} parameter) predicted by the model was only 0.396% and occurred as a well-defined peak when volunteers emerged just after the crop (11 GDD). As the emergence timing of volunteers became less synchronous with the crop, PMGF frequencies declined dramatically, such that the model predicted very low frequencies of PMGF where volunteers emerged greater than 50 GDD before or 75 GDD after the spring wheat crop

TABLE 5.2 Model parameter estimates and goodness of fit statistics calculated for predicted PMGF between imazamox-resistant volunteer spring wheat (CDC Imagine) and cropped wheat (AC Barrie) as a function of volunteer density and time of emergence relative to the crop.

		Goodness of Model Fit			
Parameter†	Estimate	Criterion‡	Estimate§	MSPE Criterion‡	Estimate (% MSPE)
a_{max}	0.396 (0.124)	F-value	93.61***	ECT	0.773
i	0.004 (0.001)	*Pseudo* R^2	0.71	ER	0.700
c	11.03 (3.54)	MSPE	0.0008	ED	98.5
f	63.9 (4.51)	rMSPE	1.68		

† Model parameter a_{max} is the amplitude of the curve, i is the percentage PMGF per unit volunteer as volunteer wheat density approaches 0 and emergence time approaches c, c is the shift of the curve peak in GDD from 0, and f is the deviation of distribution from curve peak (spread of the curve).

‡ MSPE = mean square prediction error; rMSPE = root of MSPE expressed as a percentage of the observed mean; ECT = error in central tendency; ER = error due to regression; ED = error due to disturbance.

§*** Significant at the 0.001 probability level.

TABLE 5.3 Variation in the multiplicative function describing PMGF between cropped and volunteer spring wheat among sites and years.

			$H_o = a_{max}$, i, c, and f do not vary		
Year	Site	Comparison made	df_1†	df_2‡	F_{calc}§¶
—	Carman	Across years	4	32	2.68 NS
—	Winnipeg	Across years	4	28	2.68 NS
2005	—	Across sites	4	28	2.93 NS
2006	—	Across sites	4	32	2.68 NS
Pooled Model		Across sites and years	12	60	1.99 NS

†df_1, numerator degrees of freedom.
‡df_2, denominator degrees of freedom.
§ F_{calc}, calculated F statistic.
¶NS, not significant.

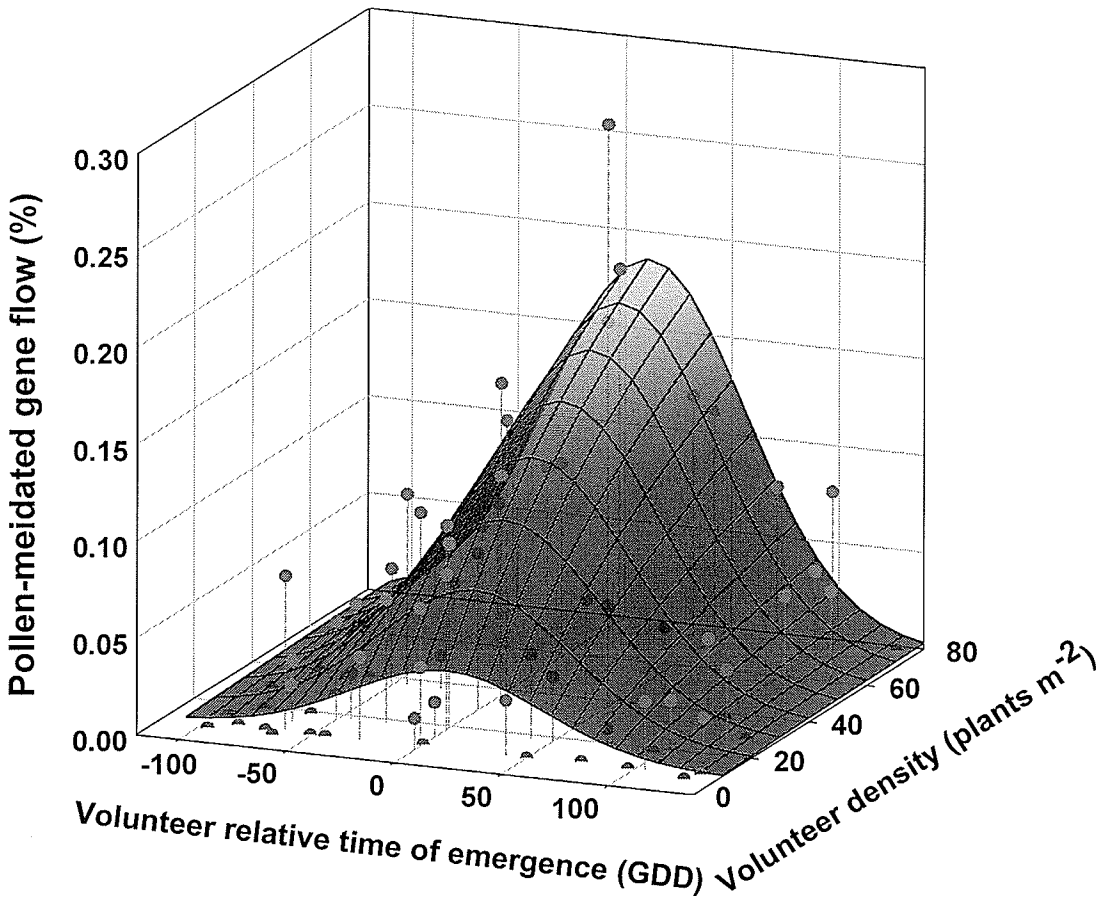

FIGURE 5.3 Pollen-mediated gene flow in spring wheat predicted as a function of volunteer wheat density and time of emergence relative to the wheat crop. Predicted values are represented by lines on the response surface and are the result of fitting the combined data to Equation 5.3. Observed values are the means of four replications and are represented by points either above (●) or below (●) the transparent response surface.

(Figs. 5.3 and 5.4). Based on the observed data, the empirical model identified a hybridization window of 125 GDD in the spring wheat crop, as a function of volunteer emergence timing (Fig. 5.4). Indeed, approximately 77% of samples with confirmed hybrids occurred within this interval. Moreover, observed mean PMGF frequencies (averaged across years and locations) were four-fold and two-fold greater when volunteer emergence coincided with the crop compared to when volunteers emerged more than 50 GDD before or 75 GDD after the crop, respectively (Table 5.1).

Pollen-mediated gene flow in spring wheat was reduced by both temporal separation and volunteer wheat density. The frequency of PMGF decreased hyperbolically with decreasing volunteer wheat density which, by holding crop density constant, served to decrease the pollinator to source plant ratio (Fig. 5.2). The predicted frequency of PMGF was always below 0.1% when volunteer wheat density was below 40 plants m^{-2} (Fig. 5.3). By contrast, PMGF frequencies increased rapidly at densities above 40 plants m^{-2} (a receptor to pollinator ratio of 6:1) to a maximum observed frequency of 0.26%. However, the rate of increase was dependent on emergence timing, with greater increases observed when volunteer emergence coincided with the crop as compared to when emergence occurred outside of the 125 GDD hybridization window. Very little gene flow was both observed and predicted at volunteer wheat densities of 10 plants m^{-2} or lower, especially outside of the hybridization window.

The frequency of PMGF showed a positive association with both flowering synchrony and the (joint) probability of synchronous flowering between volunteer and cropped spring wheat (Table 5.4). Although these correlations were significant ($P < 0.01$), they were only moderate in magnitude ($r = 0.41$ to 0.43). Moreover, linear regression revealed that flowering synchrony and joint probability explained only 18% and 16%, respectively ($P < 0.001$), of the variation in PMGF data (Fig. 5.5). Correlations between PMGF, grain yield, and yield components were weak and insignificant in all cases (Table 5.4). This is not surprising considering that grain yield and yield components also were not significantly affected by emergence timing or density of volunteers (data not shown).

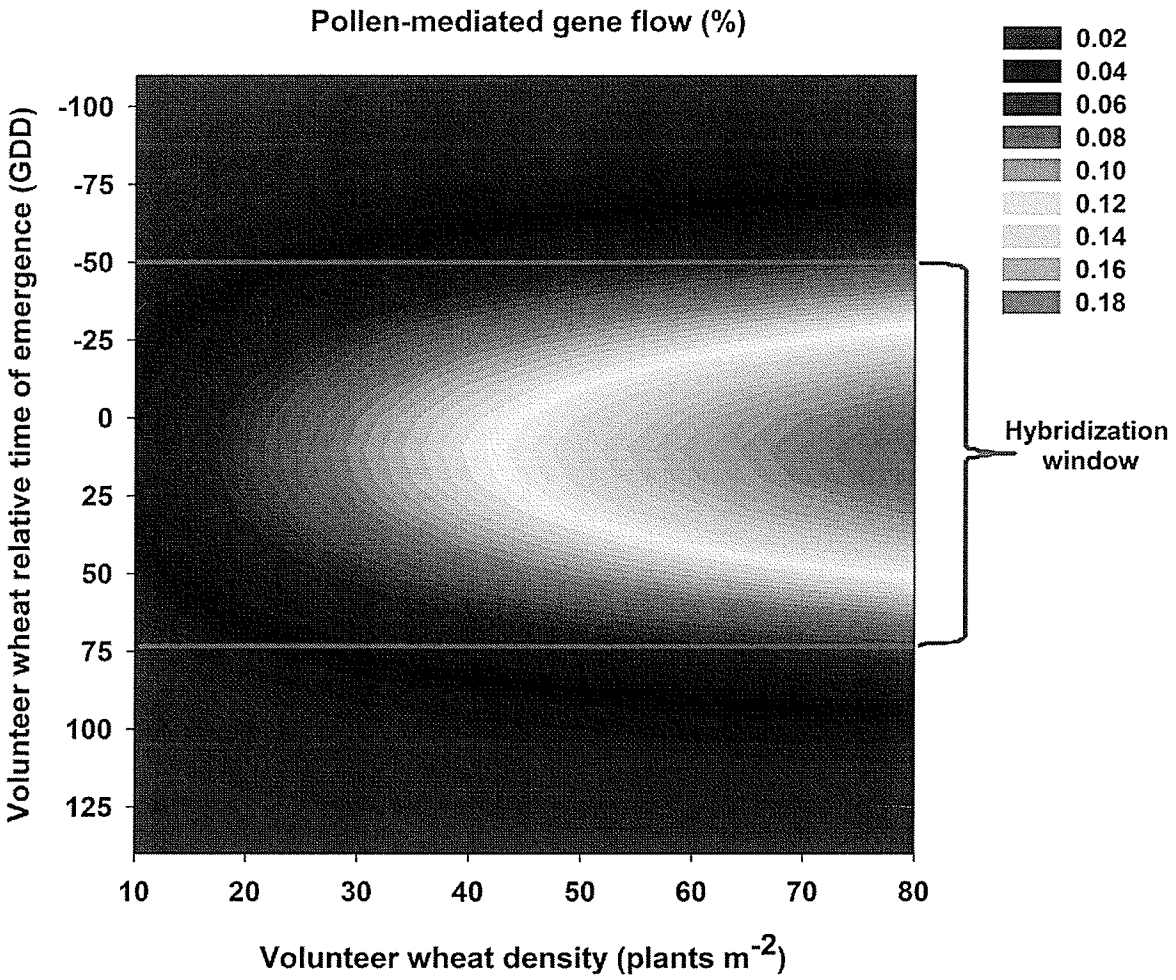

FIGURE 5.4 A contour plot of predicted PMGF (combined data fit to Equation 5.3) showing a hybridization window 125 GDD (Tbase 0.4°C) long. Emergence outside of this window resulted in dramatic reductions in PMGF frequencies between volunteer and cropped wheat.

TABLE 5.4 Correlations between flowering synchrony, joint probability, gene flow, and several response variables for spring wheat plants in 2005 and 2006.

Variable	Flowering Synchrony†	Joint Probability†	Gene flow†	Spikes plant^{-1}†	Seeds spike^{-1}†	Grain yield
Flowering synchrony	-	-	-	-	-	-
Joint probability	0.96***	-	-	-	-	-
Gene flow	0.43***	0.41**	-	-	-	-
Spikes plant^{-1}	-0.01	-0.12	0.21	-	-	-
Seeds spike^{-1}	**0.18**	**0.04**	**0.17**	**0.34****	-	-
Grain yield	0.19	0.04	0.12	0.86***	0.66***	-

†*,**,*** Correlations significant at the 0.05, 0.01, and 0.001 probability levels, respectively. Values given are Pearson correlations except those in bold type, which are Spearman Rank correlations that were employed when residuals were non-normal.

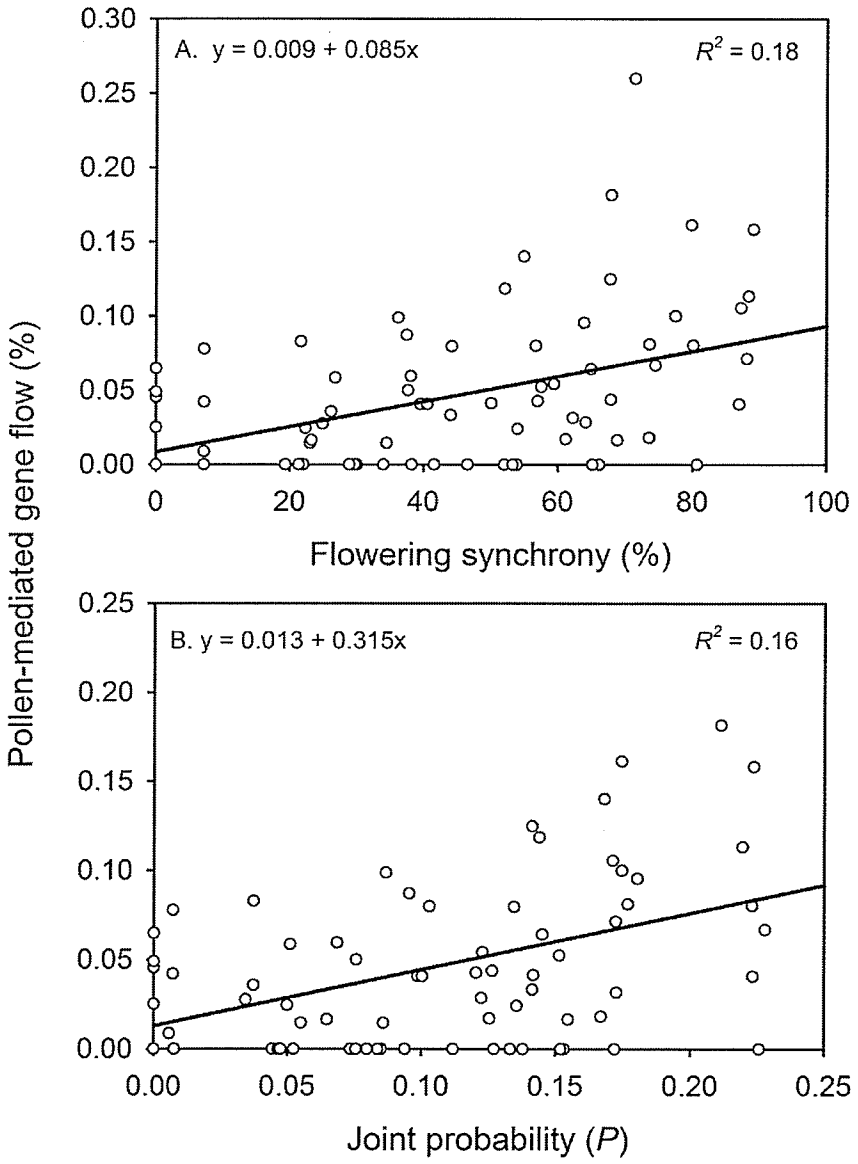

FIGURE 5.5 Linear regressions for spring wheat PMGF vs. flowering synchrony (A), and PMGF vs. the probability (joint distribution) of synchronous flowering (B). Regressions were conducted on combined data with symbols (○) representing the observed means of four replications.

As expected, a statistically significant and strong correlation was observed between flowering synchrony and joint probability because they were calculated from the same data. Spring wheat grain yield was also, not surprisingly, highly positively correlated with spikes plant^{-1} and seeds spike^{-1}.

5.4 Discussion

This study demonstrated relatively low frequencies of PMGF between a spring wheat crop and a volunteer wheat population present within it, based on both observations and model analyses. The maximum observed frequency of PMGF was 0.26%, and model analyses predicted a maximum of 0.40% (Fig. 5.3, Table 5.2). These findings are consistent with previous reports of close-quarters outcrossing in wheat (Martin, 1990; Hucl, 1996; Matus-Cádiz et al., 2004; Hanson et al., 2005b). Therefore, the frequency of PMGF in spring wheat is expected to be much lower than the 0.9% threshold established by the European Union. However, the frequency of PMGF observed in our study should not be dismissed given that even low initial frequencies of PMGF can have profound implications with adequate selection pressure for and the continued migration of a transgene (Brûlé-Babel et al., 2006). The frequencies of PMGF reported here are well above the 0% desired by some producer and consumer groups. Thus, our results demonstrate that it may not be possible, even with temporal isolation, to ensure the total absence of transgenic impurities in nontransgenic wheat. Matus-Cádiz et al. (2004) have suggested tolerance levels of 1% to 5% which, with the appropriate temporal isolation from volunteer wheat populations and neighboring crops, could be met based on the results of this study.

Time and distance may be employed together to limit PMGF, and increasing one factor reduces the requirement for the other in order to achieve defined levels of trait confinement (Halsey et al., 2005). In the case of spring wheat, however, many nontransgenic wheat crops will be grown adjacent to transgenic fields or will contain volunteer wheat populations intermixed within. These situations will demand temporal isolation of reproduction to meet predefined transgenic impurity thresholds. This study

has shown that even in the extreme case of a high proportion of volunteer wheat plants (80 plants m^{-2}), the greatest frequency of confirmed IR hybrids in the wheat crop outside of the 125 GDD hybridization window was <0.1% (Fig. 5.3). Moreover, while measurable PMGF was detected across the entire range of volunteer wheat emergence timings, peak PMGF when volunteer and cropped wheat emergence coincided was four-fold greater than when emergence occurred outside the hybridization window (Table 5.1). Thus, this study has identified a hybridization window in spring wheat that facilitates dramatic reductions in PMGF frequencies regardless of volunteer wheat density (Fig. 5.4). Because temporal separation is dependent on the accumulation of heat units which drive crop growth, the results reported here cannot be interpreted on a chronological time scale. However, spring wheat phyllochron intervals range between 80 to 100 GDD (Bauer et al., 1984; Shirtliffe et al., 2000) and therefore, we can expect wheat plants possessing one more or one less leaf to have emerged (and probably flower) within the hybridization window (Chapter 4; Willenborg et al., 200x).

The hybridization window will function to reproductively isolate not only volunteer wheat populations emerging within a 125 GDD interval of the wheat crop, but also adjacent crops emerging within this window as well. Based on our results, we suggest that temporal isolation could be a very effective mechanism to minimize both pollen-mediated crop-to-crop and volunteer-to-crop gene flow in wheat fields. Of course the exact magnitude of PMGF will be genotype-dependent, but ensuring that emergence of neighboring crop plants or coexisting volunteers does not occur within 125 GDD of crop emergence will minimize PMGF regardless of genotype. This conclusion agrees well with Halsey et al. (2005) who proposed that temporal isolation was an important mechanism to minimize outcrossing in corn, effectively reducing the separation distance necessary to achieve genetic isolation. Likewise, Della-Porta et al. (2008) reported a 50% reduction in transgene flow among corn plots when flowering dates differed by 6 d. In the current study, an offset of 3 to 5 d in flowering dates reduced the frequency of PMGF by between 200% and 400% (Table 5.1). However, for volunteer populations that are commonly present within wheat fields, transgene escape through seed-mediated gene

flow will be unavoidable. Therefore, a thorough risk assessment of seed-mediated gene flow between cropped volunteer wheat is still required.

This experiment also showed that PMGF frequencies were only marginally associated with flowering synchrony (Table 5.4). Combining the results in Chapter 4 with those presented here allows us to demonstrate that both peak flowering synchrony and PMGF are observed when volunteer and cropped wheat emergence coincides. Not surprisingly, a similar response to emergence timing was observed for both flowering synchrony and PMGF. Nevertheless, correlations between PMGF and flowering synchrony, although significant, were moderate in magnitude. Regression between the two variables explained only a small proportion of the variability (18%) in the PMGF data. This discrepancy likely resulted from the extended stigma receptivity of wheat plants, which are known to be receptive to pollen for up to 13 d (De Vries, 1971; Khan et al., 1973). Thus, flowering synchrony can provide a general indication of the potential for PMGF, but cannot be used to accurately predict PMGF frequencies in spring wheat.

In conclusion, temporal isolation should maintain pollen-mediated volunteer-to-crop and crop-to-crop gene flow in spring wheat at negligible levels, although seed dispersal still represents a major mechanism for gene flow. Strict control of volunteers emerging within a 125 GDD hybridization window will be critical to maintaining low frequencies of PMGF in spring wheat fields. However, because producers cannot selectively eliminate volunteer plants emerging after the wheat crop some level of gene flow from volunteer populations will be inevitable. In the case of crop-to-crop gene flow, it will be difficult and impractical to coordinate sowing dates among producers. However, given sufficient temporal (emergence offset by 100 GDD) and spatial isolation (minimum 45 m; Hanson et al., 2005b), it should be possible to meet reasonable tolerance levels of 1% to 5% as suggested by Matus-Cádiz et al. (2004). Nevertheless, our study confirms that temporal isolation is an effective way to confine pollen from IR wheat, and we show that a small hybridization window of only 125 GDD can be expected to dramatically reduce PMGF frequencies in transgenic spring wheat crops. Provided intense selection pressure is not continuously imposed, such low frequencies of volunteer-to-crop PMGF should rarely cause seed impurity problems. Based on our results, strict control of volunteer

populations will be necessary to keep volunteer populations low as volunteer density can exacerbate the effect of emergence timing on PMGF. Further studies should test for similar results using a range of cultivars and densities, but we expect that our general findings will hold true. Further research should also be directed at coupling temporal isolation with spatial isolation to achieve even greater reductions in spring wheat PMGF. Finally, the model developed here based on PMGF in spring wheat may also be useful in predicting PMGF in other important self-pollinated crops including rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.).

6.0 Crop genotype and density influence flowering phenology and synchrony between volunteer and cropped spring wheat

6.1 Introduction

When it comes to plant reproduction, timing is everything. Fundamental to seed production are the timing and propensity of flower production and abortion. Premature flowering can produce individuals with limited seed production capacity while delayed flowering may result in failure to produce viable seed before season's end (Elzinga et al., 2007). Similarly, the transfer of genes from one plant to another is also inherently dependent upon flowering characteristics. Date of floral initiation, flowering duration, and propensity of flowering collectively determine an individual's flowering phenology (Schmitt et al., 1987). Flowering phenology is considered a key characteristic in plant reproductive biology because it strongly influences both the timing and success of plant reproduction, either directly or indirectly. Collectively, the flowering phenologies of populations exhibit aggregate temporal properties and spatially explicit flowering abundance patterns, which are derived from the distributions on individual phenological traits (Rathcke and Lacey, 1985). Thus, modifications to an individual's flowering phenology can affect important parameters such as synchronicity of flowering at the population level and ultimately, the transfer of genes between individual plants or populations.

Transgenic crops have been widely adopted over the past decade, and their cultivation continues to increase at a sustained pace (James, 2007). The acreage devoted to transgenic crops increased 12% between 2006 and 2007, with an estimated 114.3 Mha

currently sown globally (James, 2007). The popularity of transgenic crops is not, however, without concern regarding the ecological and agricultural quandaries associated with their cultivation. Of particular importance with regard to risk assessment, property rights protection, and liability is the migration of transgenes to the same species (intraspecific gene flow) including neighboring non-transgenic crops and volunteer crop plants (Brûlé-Babel et al., 2006). While many conditions need to be satisfied to ensure successful intraspecific and interspecific gene flow, flowering phenology and more specifically flowering synchrony, are thought to be major determinants (Simard and Légère, 2004).

Transgenic wheat has been proposed for wide-scale cultivation, but no commercial releases have occurred to date. Several traits have, however, been introduced into wheat using recombinant DNA technologies (Janakiraman et al., 2002; Wilson et al., 2003) and the eventual release of transgenic wheat genotypes necessitates a thorough examination of gene flow in wheat. Gene flow is a naturally occurring biological process facilitated by pollen and seed dissemination; in flowering plants, however, pollen dispersal is often the main mode of gene flow (Levin and Kerster, 1974). Therefore, a systematic characterization of the reproductive biology and flowering phenology of wheat plants should be carried out prior to large-scale adoption of transgenic cultivars to provide a comprehensive understanding of the factors that collectively facilitate gene flow (Waines and Hegde, 2003).

The majority of wheat florets are hermaphroditic, but unisexual flowers can be identified near the terminal and distal ends of the spike (De Vries, 1971). As a result, wheat is predominantly self-pollinating and generally exhibits low levels of pollen-mediated gene flow (PMGF) under field conditions, usually below 1% (Poehlman and Sleper, 1995; Waines and Hegde, 2003). The percentage of extruding anthers in the florets of a spikelet can range from 25 to 80% among several wheat genotypes (Joppa et al., 1968). Hucl (1996) reported that flowering duration among 10 Canadian spring wheat genotypes ranged from 3.1 to 3.7 d, whereas differences in outcrossing ranged from 0.3 to 5.4%. The earliest and latest flowering genotypes varied in date of floral initiation by 7 to 8 d. While no single spike characteristic was associated with gene flow, it is important

to note that flowering synchrony between donor and recipient populations was not measured (Hucl, 1996). Genotypes with a large number of open florets before anther dehiscence and/or a longer flowering duration may be more prone to outcrossing than genotypes with a shorter flowering duration, depending on flowering synchrony levels (Willenborg and Van Acker, 2008).

Genotypic differences in flowering phenology may also be associated with variation in plant height. Semi-dwarf genotypes may be shaded by taller wheat volunteers (from a previous wheat crop). Shifts in the red:far-red ratio are known to stimulate flowering (Ballaré, 1999; Smith, 2000), and therefore may result in differences in PMGF. Martin (1990) observed significantly higher PMGF in semi-dwarf as compared to tall winter wheat genotypes, but reported that height was not correlated with gene flow. Conceivably, increasing crop density could also affect flowering phenology by increasing competition for resources and/or reducing the number of tillers per plant. In wheat, the spike of the main stem flowers first, followed in turn by the spikes of tillers in the order of their origin (Turner et al., 1994). Accelerated flowering at increased plant population densities has been demonstrated in numerous plant species (Palmblad, 1968; Schmitt et al., 1987).

Relatively little is known about the flowering phenology of spring wheat and synchrony with volunteer populations, and the impact of agronomic factors on phenological characteristics. The current study was initiated to determine the influence of crop density, genotype, and height on flowering phenology and synchrony between volunteer and cropped spring wheat populations.

6.2 Materials and Methods

6.2.1 Site Description

A field experiment was conducted at Carman, MB, Canada, (49°29′N, 98°00′W) and Winnipeg, MB, Canada, (49°68′N, 97°11′W) from 2005 to 2006. The experiment was

situated on different plot areas each year and was sown on either canola (Carman) or flax stubble (Winnipeg). The soil type at Carman was an Udic Haploboroll, Eigenhof Series sandy loam (46% sand, 24% silt, 30% clay) with a pH of 5.9 and 7.5% organic matter. The soil type at Winnipeg was a Boralfic Haploborol clay (20% sand, 26% silt, 55% clay) with a pH of 6.9 and 9.1% organic matter.

Prior to sowing, fertilizer was broadcast evenly across the entire plot area based on soil test recommendations. The experimental area at Carman received a tillage pass in the fall with a field cultivator and a second tillage operation was used to incorporate fertilizer in the spring. The site at Winnipeg received only a single tillage pass with a heavy harrow immediately prior to sowing to prevent excessive formation of soil clods. Plot areas at both sites were chosen on the basis of previous crop history, with a requirement of at least two years since any previous wheat crop to ensure low native volunteer wheat populations. Both sites received a glyphosate application of 900 g a.e. ha^{-1} immediately prior to sowing to control emerged weeds.

6.2.2 Experimental Approach

The experimental design consisted of a factorial arrangement of two treatment variables (crop genotype and density) in a split-plot design with four replicates. The flowering phenology and synchrony of two tall spring wheat genotypes (cv. Prodigy, Graf et al., 2003; cv. Amazon, Anon., 2005c) were compared with two semi-dwarf genotypes (cv. Oslo, Graf et al., 1990; cv. AC Vista, DePauw et al., 1998) across four target crop seeding densities (75, 150, 300, and 600 plants m^{-2}). Genotypes were chosen based on diversity of pedigrees and height, but were similar in maturity to the volunteer genotype (Manitoba Agriculture, Food, and Rural Initiatives, 2008). Crop density was the main plot factor and was randomized among replicates whereas subplots were composed of various crop genotypes and were 2 by 6 m in length.

Volunteer wheat (cv. CDC Imagine) was planted at a constant target density of 30 plants m^{-2}. Prior to sowing, thousand kernel weight and germination tests were used to determine seeding rate. Volunteer and cropped wheat were planted at a depth of 2 cm in

one pass in alternating 15.2 cm rows using a low disturbance, double-disc plot drill. Seeding dates in 2005 and 2006 were 11 May and 9 May at Carman and 16 May and 12 May at Winnipeg, respectively. A tank-mix of clodinafop-propargyl at 56 g a.i. ha^{-1}, thifensulfuron-methyl plus tribenuron-methyl (1:1) at 7 g a.i. ha^{-1}, bromoxynil plus MCPA ester at 27 g a.i. ha^{-1} with Score adjuvant (1% v/v) was used for post-emergence weed control at Carman, while the same tank-mix with the addition of 445 g a.i. ha^{-1} fluroxypyr for volunteer flax control was applied at Winnipeg. Preventative disease control was achieved using an application of 124 g a.i. ha^{-1} of propiconazole in all site-years. Plots at Carman also received an application of 200 g a.i. ha^{-1} dimethoate for control of aphids in 2006.

Volunteer and cropped wheat emergence were determined by counting the number of emerged plants in two 1 m rows 3 to 4 wk after sowing when plants were in the two- to three-leaf stage. The flowering period was quantified by rating flowering daily on the Zadok's Scale (Zadoks et al., 1974) (based on the proportion of extruding anthers) on each of 10 randomly selected spikes per plot for both volunteer and cropped wheat plants. This rating provided a number of estimates for both the volunteers and crop including mean days to 5%, 50%, and 95% flowering (calculated below from Equation 6.2), flowering period (calculated as mean days to first and last flower), and flowering overlap between volunteers and crop. Plant height of cropped and volunteer wheat at flowering was determined by measuring the height of six plants per plot. At physiological maturity, a subsample of crop plants in a 1 m row were cut from each plot and the number of fertile tillers (spikes) was recorded.

6.2.3 Statistical Analysis

Flowering phenology was characterized by first fitting data to a nonlinear, increasing sigmoidal equation as a function of time using the nonlinear regression procedure of SAS, with iterations derived by the Gauss-Newton algorithm (SAS Inst., 2004). Flowering ratings were expressed as a percentage and fit to the four parameter logistic equation give by:

$$P_f = C + D/\left(1 + \left(x/P_{50}\right)^b\right) \tag{6.1}$$

where P_f is the percentage flowering at time x, x is time in Julian days, $C + D$ is the upper asymptote, C is the lower asymptote, b is the slope, and P_{50} is the x value (Julian date) at the midpoint of the curve. The product of this nonlinear regression procedure is a flowering progress curve, which served to facilitate further analysis. Temporal variation in flowering was characterized by estimating the time required to obtain 5, 50, and 95% flowering for each cropped wheat genotype and density as well as volunteers utilizing the following equation:

$$T_f = P_{50} \cdot \left(\left(D/\left(y - C\right)\right) - 1\right)^{\frac{1}{b}} - D_s \tag{6.2}$$

where T_f is cumulative time from sowing to 5, 50 or 95% flowering (days), y is percentage flowering, D_s is date of sowing (Julian date), and P_{50}, b, C, D are the nonlinear parameters described above for Equation 6.1. For comparisons, time from sowing to each level of flowering was expressed both in days and growing degree days (GDD), which were calculated as the cumulative summation of daily average temperature as in Willenborg et al. (2005), with a base temperature of 0.4°C (Addae and Pearson, 1992) and a maximum temperature of 30°C (Cao and Moss, 1989) assumed for wheat growth.

The distribution of flowering times (Equation 6.1) represents a cumulative density function (CDF). Therefore, the degree of overlap among curves can be assessed by calculating the probability density function (PDF) for each population and assessing the degree of overlap between the two distributions. This can be expressed mathematically as:

$$f(x) = \frac{dF(x)}{dx} = -\frac{D\left(\frac{x}{P_{50}}\right)^b b}{\left(1 + \left(\frac{x}{P_{50}}\right)^b\right)^2 x} \tag{6.3}$$

where $f(x)$ is the first derivative of the sigmoidal function (or rate of flowering of the population expressed as percent flowering per day), and b, D, P_{50}, and x are the nonlinear parameters described for Equation 6.1. Equation 6.3 represents the proportion of plants

flowering on a daily basis. It can also be used to calculate the degree of overlap between different populations and most importantly, the dates of peak flowering for various crop genotypes and densities. From the genotype/density-specific distribution, the degree of overlap can be interpreted to be the level of flowering synchrony between any two populations. Numerically, the overlapping area can be calculated by integrating over the minimum value and normalizing by the integral over the entire PDF of either (both are one by definition of a probability distribution). Symbolically:

$$F_s = \int_{\infty}^{0} \mathrm{Min}\{f(x), g(x)\}\,\mathrm{d}\,x \tag{6.4}$$

where F_s is the proportion of area in common between two curves (flowering synchrony), and $f(x)$ and $g(x)$ are the cumulative flowering proportion (x) at each time interval for any two crop genotypes or densities f and g, respectively. All model calculations were performed using the Mathematica 6.0 platform (Wolfram Research, 2007). Flowering synchrony values were expressed as percentages and complete synchrony occurs if F_s = 100. Because of the stochastic nature of synchronous flowering, it was desirable to be able to predict synchronicity of flowering on the basis of crop genotype and density. To enable such predictions, a joint distribution was calculated by evaluating the integral of the product of two PDFs:

$$J = \int_{\infty}^{0} f(x) \cdot g(x) \tag{6.5}$$

where J is the joint probability of the two "events" (described by the two PDFs) occurring simultaneously, and f and g are as described in Equation 6.4.

An initial exploratory analysis was conducted using Levene's test and the UNIVARIATE procedure of SAS (SAS Inst., 2004) to determine whether residuals conformed to the assumptions of analysis of variance. Because residuals were found to be heterogeneous between locations, years, and treatments, all analyses were conducted within site-years. Residuals within site-year combinations were generally random, homogenous, with a normal distribution about a mean of zero. While spikes per plant required a logarithmic transformation, untransformed data were used in cases where transformation failed to improve the distribution of residuals. Because the negative effects of heterogeneity are often more consequential to analysis of variance than non-

normal distributions (Littell et al., 2002), heterogeneity between treatments was modeled using the GROUP option in the REPEATED statement within the mixed-model procedure in SAS (SAS Inst. 2004), and the WELCH option in the MEANS statement when evoking PROC GLM . Flowering phenology data and spikes per plant were subjected to a two-way analysis of variance within site-years using the mixed model procedure of SAS (Littell et al. 1996), with degrees of freedom calculated by Satterthwaite's approximation method. Block and its interaction with density comprised the random effects in the model whereas density, genotype, and their interactions were considered fixed effects. Because flowering synchrony values and joint probabilities were derived from nonlinear regression analysis conducted across treatments, these values were subjected to analysis of variance within site-years using PROC GLM in SAS (SAS Inst. 2004). Throughout the study, ESTIMATE statements were used to make comparisons among densities while Tukey's honestly significant difference (Tukey, 1953) was used to compare cultivar means. Pearson correlations were used to correlate flowering synchrony and joint probability with plant height and spikes m^{-2}.

6.3 Results

Contrasting growing-season weather patterns were observed at both sites in 2005 and 2006 (Table 6.1). In 2005, precipitation was well above-average from May to July at both locations. In contrast, both locations experienced below-average levels of precipitation in nearly every month between May and September, 2006. Temperatures were close to normal at Carman and Winnipeg in 2005 but above-average at both sites in 2006.

The four spring wheat genotypes included in this study varied significantly ($P < 0.001$) with respect to plant height in all site-years (Table 6.2). Amazon and Oslo were the tallest and shortest of the genotypes included in the study, respectively, while Prodigy and Vista were intermediate (Fig. 6.1A). Although the magnitude of the differences in height between genotypes varied in any given site-year, rankings were always consistent: Amazon > Prodigy > Vista > Oslo. With the exception of Carman in 2006, plant height of individual genotypes varied little among years and locations, suggesting that plant height

TABLE 6.1 Monthly precipitation (mm), mean daily temperature (°C), and the long-term (30-yr) average of each for Carman and Winnipeg, MB, Canada in 2005 and 2006. Thirty-year averages were obtained from Environment Canada (2008).

		Precipitation (mm)			Temperature (°C)		
Location	Month	2005	2006	30-yr average	2005	2006	30-yr average
Carman	April	19.0	34.3	33.4	7.1	8.5	4.2
	May	88.0	28.0	53.4	10.2	12.4	12.5
	June	139.8	50.3	81.0	17.4	17.9	16.9
	July	89.6	26.2	71.1	19.7	20.4	19.4
	August	23.6	37.4	70.0	17.6	18.8	18.2
	September	31.2	42.8	57.7	14.3	13.2	12.3
	Total	391.2	219.0	366.6	—	—	—
Winnipeg	April	29.0	6.5	31.9	7.4	9.4	4.0
	May	68.8	91.2	58.8	10.8	12.8	12.0
	June	170.7	20.3	89.5	17.9	19.2	17.0
	July	191.5	14.2	70.6	20.8	22.4	19.5
	August	22.0	73.4	75.1	18.5	19.9	18.5
	September	46.2	40.6	52.3	14.8	13.8	12.3
	Total	528.2	246.2	378.2	—	—	—

TABLE 6.2 Significance (P) of the effect of genotype, density, their interaction, and trend comparisons on cropped and volunteer spring wheat height, height differential, and number of fertile crop tillers per plant (spikes m^{-2}). P values are in bold font where significant at $P \leq 0.05$.

		P value			
Site-year	Source	Crop height	Volunteer height	Height differential	Crop spikes m^{-2}
Carman 2005	Genotype (G)	**0.000**	0.121	**0.000**	0.227
	Tall vs. Short	**0.000**	0.565	**0.000**	0.790
	Density (D)	0.157	0.361	0.227	**0.000**
	Linear				**0.000**
	G X D	0.090	0.869	0.724	0.487
Winnipeg 2005	Genotype (G)	**0.000**	0.167	**0.000**	0.175
	Tall vs. Short	**0.000**	0.056	**0.000**	0.881
	Density (D)	0.565	0.367	0.195	**0.001**
	Linear				**0.000**
	G X D	0.304	0.576	0.794	0.956
Carman 2006	Genotype (G)	**0.000**	**0.015**	**0.000**	0.103
	Tall vs. Short	**0.000**	0.223	**0.000**	0.175
	Density (D)	**0.020**	0.994	0.067	**0.000**
	Linear				**0.000**
	G X D	0.121	0.667	0.701	0.394
Winnipeg 2006	Genotype (G)	**0.000**	0.088	**0.000**	0.405
	Tall vs. Short	**0.000**	0.731	**0.008**	0.377
	Density (D)	0.057	0.616	**0.045**	**0.000**
	Linear			**0.017**	**0.000**
	G X D	0.210	0.912	0.635	0.340

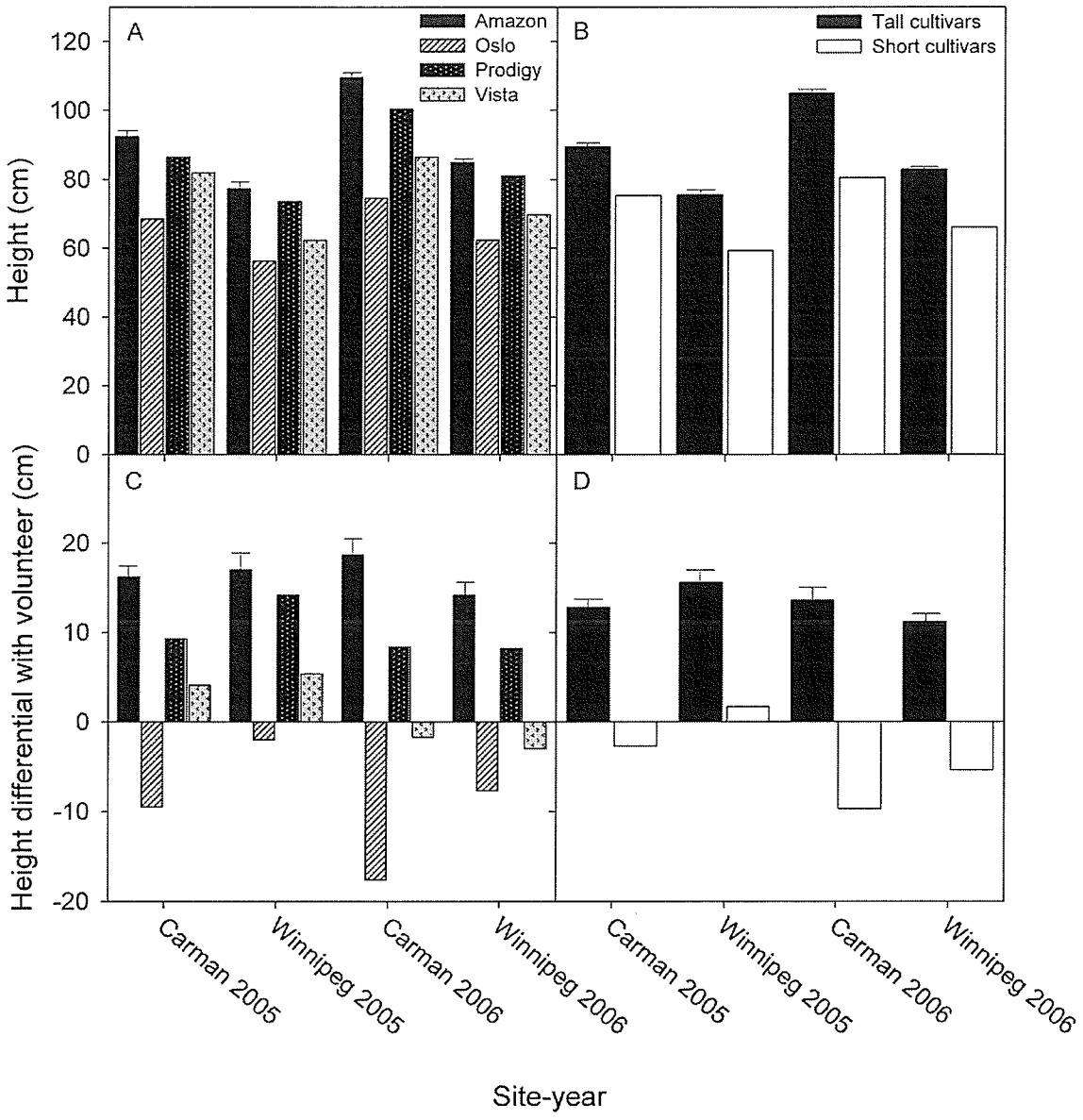

FIGURE 6.1 Relationships between crop height (A) and the height differential between cropped and volunteer spring wheat (C) as a function of crop genotype. Mean differences between tall vs. short genotypes for height (B) and height differential (D) as derived from single degree of freedom contrasts. Field studies were carried out at Carman and Winnipeg, MB, Canada, in 2005 and 2006. Bars indicate ± 1 standard error of the difference between means.

for these genotypes was only marginally sensitive to environmental conditions. Tall genotypes (Amazon and Prodigy) were always significantly ($P < 0.001$) taller than semi-dwarf genotypes (Oslo and Vista), with the mean difference between the two averaging 17.2 cm among site-years (Fig. 6.1B). Similarly, differences in height between the crop and volunteer plants (height differential) varied between genotypes in any one site-year (Table 6.2). As expected, mean plant height (averaged across site-years) for the tall genotypes Amazon and Prodigy was 16.5 and 10 cm greater than the volunteer, while for the semi-dwarfs Vista and Oslo it was 1.2 cm greater and 9.2 cm shorter than the volunteer, respectively (Fig. 6.1C). Consequently, the semi-dwarfs were shaded by the volunteers in most site-years (Fig. 6.1D). Crop density generally had no significant ($P \leq 0.05$) effect on plant height for either the crop or the volunteers (Table 6.2).

Genotype effects on flowering phenology were evident for all variables measured (Table 6.3). Although three significant ($P \leq 0.05$) interactions were observed between density and genotype, they accounted for only a very small proportion of the total sums of squares (1–5%) and therefore, were largely inconsequential to statistical outcomes. Flowering onset occurred for Olso 1 to 5 d (19 to 81 GDD) earlier than for any other genotype in three of four site-years (Table 6.4, Fig. 6.2). Despite being statistically significant ($P \leq 0.001$), differences in flowering onset among the other genotypes were minimal with only 1 d separating the onset of flowering in the later flowering genotype Amazon from Prodigy and Vista (Table 6.4). Mean thermal time to flowering onset, averaged across site-years and genotypes, was approximately 900 GDD.

These genotypic differences persisted throughout the length of the flowering period (Table 6.3, Fig. 6.2). Time to 50% and 95% flowering also occurred ($P \leq 0.001$) earlier for Oslo than for any other genotype (Table 6.4). Oslo required an average (among site-years) of only 915 GDD to reach the midpoint of the flowering period as compared to 975 GDD among the other genotypes. Likewise, crop flowering period duration differed ($P \leq 0.01$) among genotypes in two of four site-years (Table 6.3), with Oslo tending to flower for 0.5 d and 0.7 d (on average) longer than Amazon and Prodigy,

TABLE 6.3 Significance (P) of the effect of genotype, density, their interaction, and trend comparisons on cropped and volunteer spring wheat time to 5, 50, and 95% flowering and flowering period duration. P values are in bold font where significant at $P \leq 0.05$.

		p value			
Site-year	Source	Time to 5% flowering	Time to 50% flowering	Time to 95% flowering	Flowering period duration
		Crop			
Carman 2005	Genotype (G)	**0.003**	**0.000**	**0.000**	**0.004**
	Tall vs. Short	**0.000**	**0.000**	**0.000**	**0.019**
	Density (D)	**0.000**	**0.000**	0.835	0.617
	G X D	0.358	**0.050**	**0.018**	0.940
Winnipeg 2005	Genotype (G)	**0.003**	**0.000**	**0.000**	0.068
	Tall vs. Short	**0.003**	**0.000**	**0.000**	**0.006**
	Density (D)	0.192	0.139	0.120	0.165
	G X D	0.560	0.413	0.532	0.664
Carman 2006	Genotype (G)	**0.000**	**0.000**	**0.000**	0.759
	Tall vs. Short	**0.000**	**0.000**	0.759	0.450
	Density (D)	**0.001**	**0.000**	0.755	0.111
	G X D	0.288	0.123	0.100	0.678
Winnipeg 2006	Genotype (G)	**0.000**	**0.000**	**0.000**	**0.000**
	Tall vs. Short	0.240	0.538	**0.001**	**0.002**
	Density (D)	**0.015**	**0.000**	**0.022**	0.670
	G X D	0.066	**0.017**	0.567	0.051
		Volunteer			
Carman 2005	Genotype (G)	0.181	**0.000**	0.164	0.617
	Tall vs. Short	0.069	**0.002**	0.498	0.648
	Density (D)	**0.045**	0.444	0.858	**0.004**
	G X D	0.850	0.553	0.390	0.650

TABLE 6.3 Continued

Winnipeg 2005	Genotype (G)	0.539	0.410	0.428	0.119
	Tall vs. Short	0.444	0.168	0.489	0.107
	Density (D)	0.587	0.310	0.175	**0.000**
	G X D	0.820	0.385	0.418	0.053
Carman 2006	Genotype (G)	0.071	0.399	0.142	0.051
	Tall vs. Short	**0.043**	0.326	**0.029**	0.063
	Density (D)	0.245	**0.027**	0.117	**0.032**
	G X D	0.930	0.208	0.298	0.121
Winnipeg 2006	Genotype (G)	**0.001**	**0.000**	0.141	0.732
	Tall vs. Short	**0.000**	**0.001**	0.368	0.700
	Density (D)	**0.010**	**0.047**	0.885	0.650
	G X D	0.157	0.182	0.481	0.581

TABLE 6.4 Crop genotype and density effects on cropped spring wheat time from sowing to 5, 50, and 95% flowering as well as flowering period duration. Values in parentheses represent accumulated thermal time from sowing in GDD.

	Genotype†				Target crop density† (plants m^{-2})			
Site-year‡	Amazon	Oslo	Prodigy	Vista	75	150	300	600
	Time to 5% flowering (d)							
CAR 05	58.1a (909)	53.5c (828)	56.6b (886)	58.5a (909)	58.0a (909)	57.4ab (886)	56.1bc (863)	55.2c (843)
WPG 05	56.8a (968)	54.1b (919)	55.9a (945)	55.6a (945)	56.3 (968)	55.8 (945)	55.0 (945)	55.4 (945)
CAR 06	53.4a (892)	50.5c (852)	53.1a (892)	52.2b (892)	53.1a (852)	53.1a (852)	51.8b (809)	51.3b (809)
WPG 06	53.0bc (935)	52.5c (916)	53.2b (935)	54.2a (956)	53.8a (935)	53.2ab (935)	53.1b (935)	52.7b (915)
	Time to 50% flowering (d)							
CAR 05	60.8c (968)	56.5d (919)	61.6b (945)	62.0a (945)	60.8a (968)	60.4b (945)	60.1c (945)	59.6d (945)
WPG 05	59.0a (1040)	56.9b (968)	58.7a (1017)	59.0a (1017)	58.6 (1017)	58.6 (1017)	58.2 (1017)	58.0 (1017)
CAR 06	55.3b (892)	53.9c (852)	55.2b (892)	55.8a (892)	55.3a (892)	55.4a (892)	55.0b (873)	54.5b (973)
WPG 06	55.3c (979)	54.4d (956)	55.9b (979)	56.7a (1004)	56.3a (1004)	55.7b (979)	55.4b (979)	55.0c (956)
	Time to 95% flowering (d)							
CAR 05	63.7b (1029)	59.6c (934)	64.5a (1051)	64.3a (1051)	63.1 (1029)	63.0 (1029)	63.1 (1029)	63.0 (1029)
WPG 05	61.3a (1085)	58.8b (1017)	61.3a (1085)	61.5a (1085)	60.7 (1061)	61.3 (1085)	60.7 (1061)	60.2 (1061)
CAR 06	57.6b (925)	56.8c (908)	57.7b (925)	58.6a (944)	57.7 (925)	57.7 (925)	57.8 (925)	57.5 (925)
WPG 06	58.4a (1041)	56.4b (1004)	59.3a (1059)	59.2a (1059)	58.8a (1041)	58.4ab (1041)	58.2bc (1041)	57.9c (1027)
	Flowering period duration (d)							
CAR 05	5.7b	6.3a	5.3b	5.6b	5.9	5.5	5.7	5.6
WPG 05	4.9	5.5	5.2	6.0	5.5	5.8	5.3	4.9
CAR 06	6.6	6.4	6.6	6.5	6.9	6.4	6.5	6.3
WPG 06	5.7b	6.6a	5.0c	5.0c	5.7	5.8	5.5	5.4

†Means within the same crop genotype or density row followed by the same lowercase letters are not significantly different ($P \leq 0.05$) by Tukey's HSD and single degree of freedom contrasts, respectively.
‡CAR 05 & 06, Carman 2005 and 2006; WPG 05 & 06, Winnipeg 2005 and 2006.

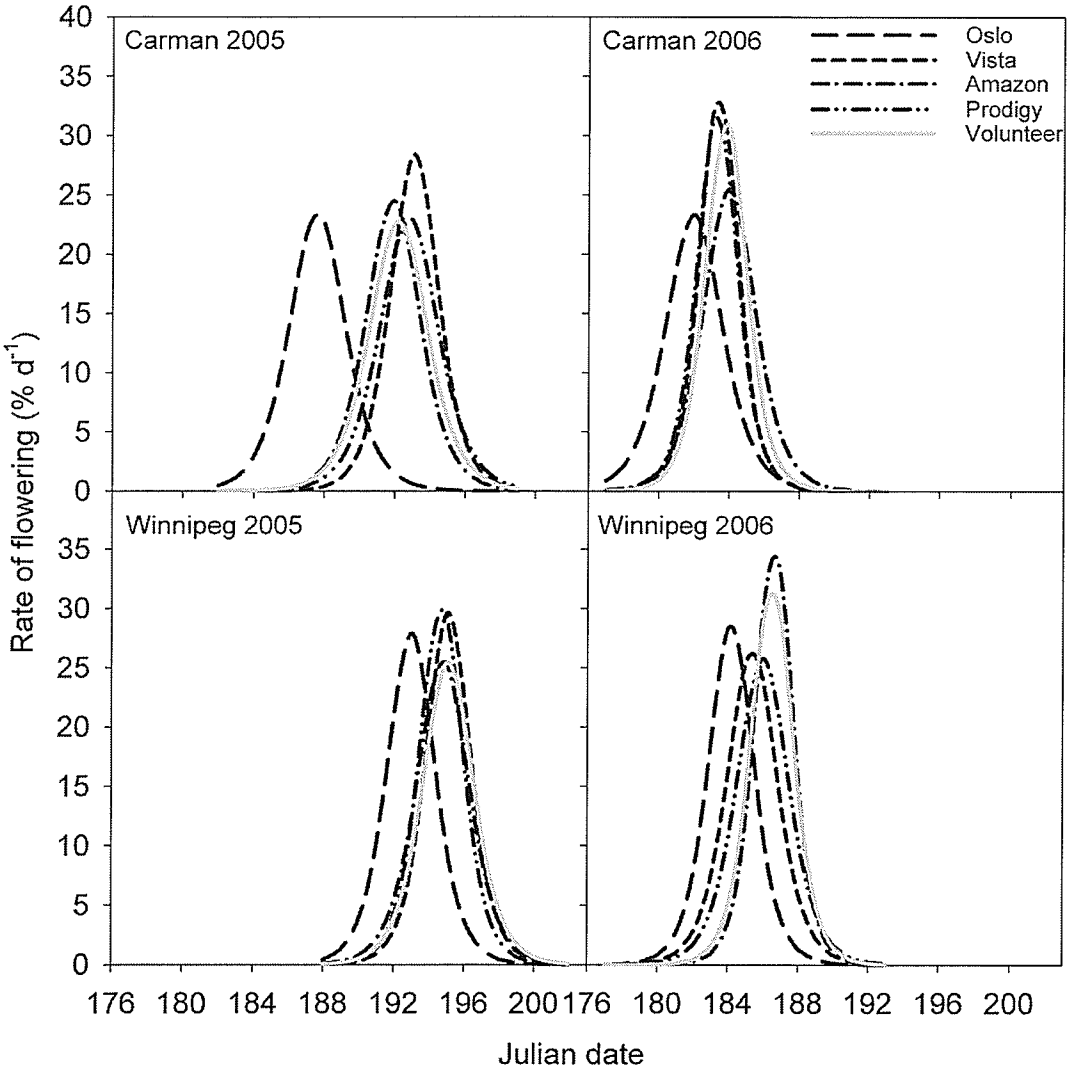

FIGURE 6.2 Rate of volunteer and cropped spring wheat flowering over time as a function of crop genotype. Field studies were carried out at Carman and Winnipeg, MB, Canada, in 2005 and 2006. Values are the result of taking the first derivative (Equation 6.3) of the logistic flowering curve (Equation 6.1) to which raw flowering data were initially fit.

respectively (Table 6.4). However, variation between genotypes was minimal compared to variation within genotypes over different site-years. In this regard, for any one genotype, little consistency existed in the timing of flowering, flowering period duration, and peak flowering (Table 6.4, Fig. 6.2). Nevertheless, early (Oslo) and late (Vista) flowering genotypes in one site-year also flowered early and late in the next. Taken together, this indicates that relative differences between genotypes remained constant regardless of the environment, but environmental effects had the greatest impact on the overall flowering period.

Crop density also affected cropped wheat flowering phenology (Table 6.3). Time to 5% flowering was significantly ($P \leq 0.05$) delayed (1 d to 3 d) at a target sowing density of 75 plants m^{-2} compared with 600 plants m^{-2} in all site-years except Winnipeg 2005 (Tables 6.3 and 6.4). Likewise, date of peak flowering in those site-years differed significantly ($P < 0.001$) between crop densities, exhibiting a near-linear decline with increasing plant density (Fig. 6.3, Table 6.4). Similar to genotypic effects, crop density had much less impact on flowering phenology than did environmental heterogeneity (Table 6.4). Time to 5 and 50% flowering varied at most by 1 to 3 d among crop densities as opposed to 2 to 11 d among site-years. Crop density did not affect either time to 95% flowering or crop flowering period duration in most site-years (Table 6.3), despite a significant near-linear decline in spikes per plant with increasing crop density (Table 6.2).

With the exception of volunteer flowering duration, the effects of both crop density and genotype on volunteer wheat flowering were marginal and inconsistent (Table 6.3). Volunteer wheat flowering duration declined at a near-linear rate with increasing crop density in three of four site-years (Table 6.5). Mean volunteer wheat flowering duration (averaged across three site-years) was 15% greater at low (75 and 150 plants m^{-2}) vs. high (300 and 600 plants m^{-2}) crop densities. Although not always statistically significant ($P \leq 0.05$), volunteer wheat plants competing with 600 crop plants m^{-2} generally exhibited a reduced time to 5, 50, and 95% flowering compared to volunteers competing with 75 crop plants m^{-2} (Tables 6.3 and 6.5).

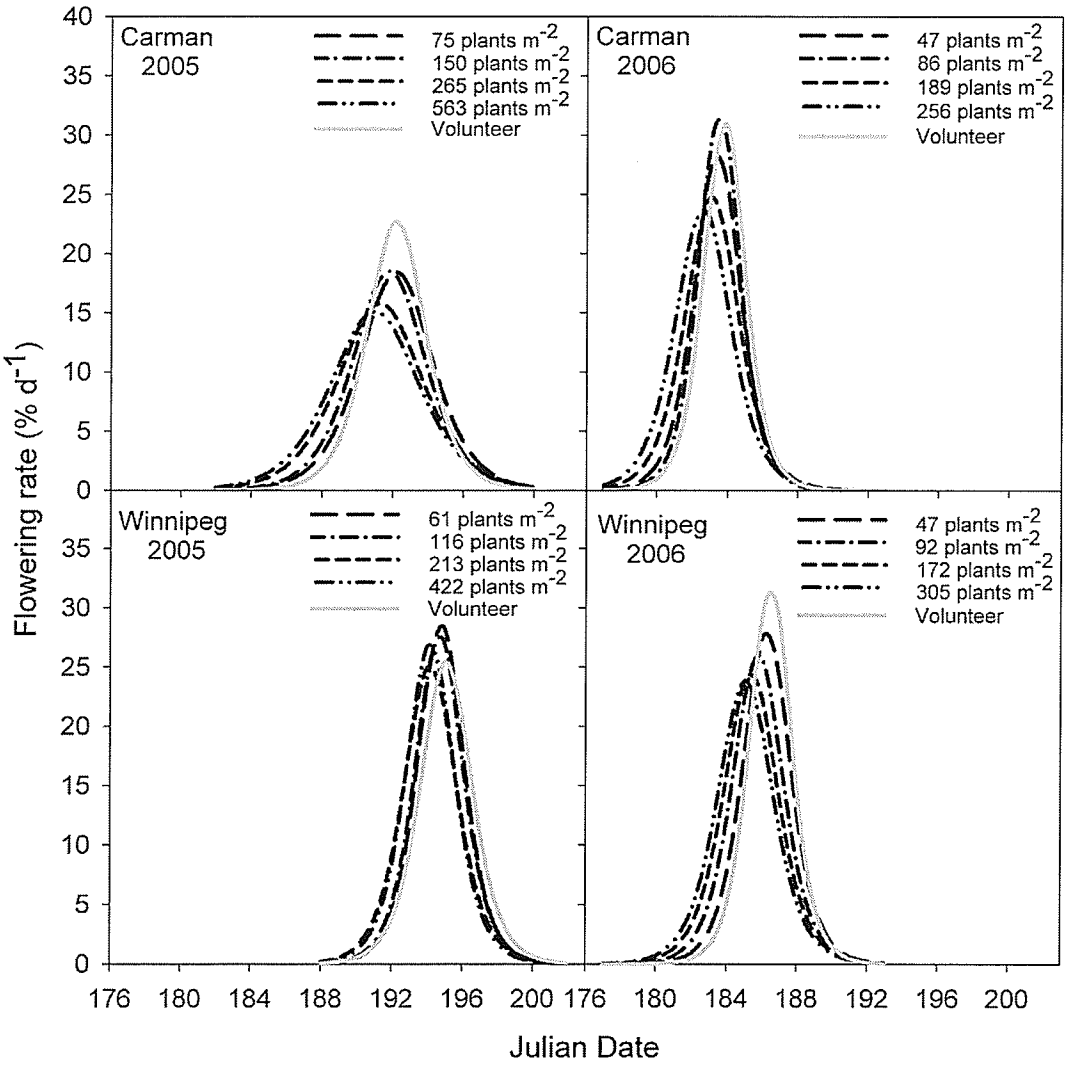

FIGURE 6.3 Rate of volunteer and cropped spring wheat flowering over time as a function of actual observed crop densities. Field studies were carried out at Carman and Winnipeg, MB, Canada, in 2005 and 2006. Values are the result of taking the first derivative (Equation 6.3) of the logistic flowering curve (Equation 6.1) to which raw flowering data were initially fit. Observed crop density means were obtained by averaging over blocks and genotypes.

TABLE 6.5 Crop genotype and density effects on volunteer spring wheat time from sowing to 5, 50, and 95% flowering as well as flowering period duration. Values in parentheses represent accumulated thermal from sowing in GDD.

	Genotype†				Target crop density† (plants m^{-2})			
Site-year‡	Amazon	Oslo	Prodigy	Vista	75	150	300	600
	Time to 5% flowering (d)							
CAR 05	58.2 (886)	56.8 (863)	57.6 (886)	57.3 (886)	58.5a (909)	57.9a (886)	57.2ab (886)	56.4b(863)
WPG 05	55.7 (945)	56.7 (968)	56.3 (968)	56.1 (968)	56.1 (968)	56.5 (968)	56.4 (968)	55.8 (945)
CAR 06	54.0 (873)	53.5 (852)	53.9 (852)	53.9 (852)	53.7 (852)	53.9 (852)	54.0 (873)	53.7 (852)
WPG 06	54.0b (935)	53.1c (935)	54.6a (956)	53.7bc (935)	54.7a (956)	54ab (935)	53.5b (935)	53.3b(935)
	Time to 50% flowering (d)							
CAR 05	61.8a (981)	60.4c (958)	61.3b(981)	61.6ab(981)	61.6 (981)	61.1 (981)	61.3 (981)	61.2 (981)
WPG 05	59.6 (1040)	59.0 (1017)	59.2(1040)	59.1 (1040)	59.4 (1040)	59.4 (1040)	59.0 (1017)	59.1(1040)
CAR 06	55.9 (892)	55.7 (892)	55.8 (892)	55.8 (892)	56.0a (908)	56.0a (892)	55.7ab (892)	55.5b(892)
WPG 06	56.7a (1004)	56.1b(1004)	56.8a(1004)	56.6a (1004)	56.9a (1059)	56.5b(1059)	56.4b (1059)	56.4b(1059)
	Time to 95% flowering (d)							
CAR 05	64.5 (1051)	63.8 (1029)	63.9(1029)	64.3 (1051)	64.1 (1051)	63.9 (1051)	64.1 (1029)	64.4(1029)
WPG 05	63.1 (1105)	62.3 (1105)	61.8(1105)	62.1 (1085)	62.9 (1105)	62.1 (1105)	62.2 (1105)	62.1(1105)
CAR 06	57.9 (925)	58.2 (944)	57.8 (925)	58.3 (944)	58.5 (944)	58.2 (944)	57.8 (925)	57.7 (925)
WPG 06	59.4 (1059)	59.2 (1059)	58.9(1059)	59.3 (1059)	59.3 (1059)	59.1 (1059)	59.2 (1059)	59.3(1059)
	Flowering period duration (d)							
CAR 05	5.9	5.7	5.6	5.6	5.9a	6.2a	5.3b	5.3b
WPG 05	6.0	6.3	5.9	5.6	6.7a	6.1ab	5.6bc	5.4c
CAR 06	5.6	6.3	6.1	6.0	6.5a	6.1ab	5.8b	5.6b
WPG 06	5.8	6.0	5.8	5.8	5.8	5.8	6.0	5.8

†Means within the same crop genotype or density row followed by the same lowercase letters are not significantly different ($p \leq 0.05$) by Tukey's HSD and single degree of freedom contrasts, respectively.
‡CAR 05 & 06, Carman 2005 and 2006; WPG 05 & 06, Winnipeg 2005 and 2006.

Flowering synchrony and the joint probability of synchronous flowering (joint distribution) between cropped and volunteer wheat populations exhibited distinct differences between genotypes (Figs. 6.4A and 6.4B). With the exception of Vista at Carman (2006), flowering synchrony and joint probability were lower ($P \leq 0.001$) for Oslo than for all other genotypes. Flowering synchrony between volunteer wheat and Oslo averaged (across site-years) 38% compared to 73% for the other genotypes. Flowering synchrony and joint probabilities between Vista and volunteer plants varied considerably among site-years (52 – 86%), whereas Amazon and Prodigy exhibited consistently high flowering synchronies (approx. 75%) and joint probabilities (approx. 0.20). Accordingly, both flowering synchrony and joint probabilities were greater (P = 0.001 – 0.009) for tall versus semi-dwarf genotypes in three site-years, the mean differences being 38% and 40%, respectively (Figs. 6.4B and 6.4D). In those same three site-years, flowering synchrony and joint probabilities were also highly correlated with height and height differentials (Table 6.6). Neither flowering synchrony nor joint probabilities responded ($P \leq 0.05$) to increasing crop density in any site-year.

6.4 Discussion

Cropped and volunteer wheat plants displayed considerable variation in flowering phenology among site-years. Reductions in the time required to achieve 5, 50, and 95% flowering were consistently noted in cropped and volunteer wheat at both sites in 2006 vs. 2005 (Tables 6.4 and 6.5). This variation was primarily driven by differences in temperature and precipitation among site-years (Fig. 6.1). Mean temperature during the flowering period was 1.2°C greater in 2006 than 2005 (Table 6.1). Flowering time in wheat is largely controlled by a quantitative response to temperature and photoperiod (Marcellos and Single, 1971), with genotypes developing more rapidly in warmer temperatures provided vernalization and photoperiod requirements are met (Halse and Weir, 1970). These findings are also consistent with other studies quantifying flowering onset in wheat. Pauli et al. (1962), for example, attributed earlier anthesis in winter wheat to higher greenhouse temperatures. Under field conditions, Matus-Cádiz et al. (2007) observed that the flowering period in the spring wheat genotype Purendo-38 increased

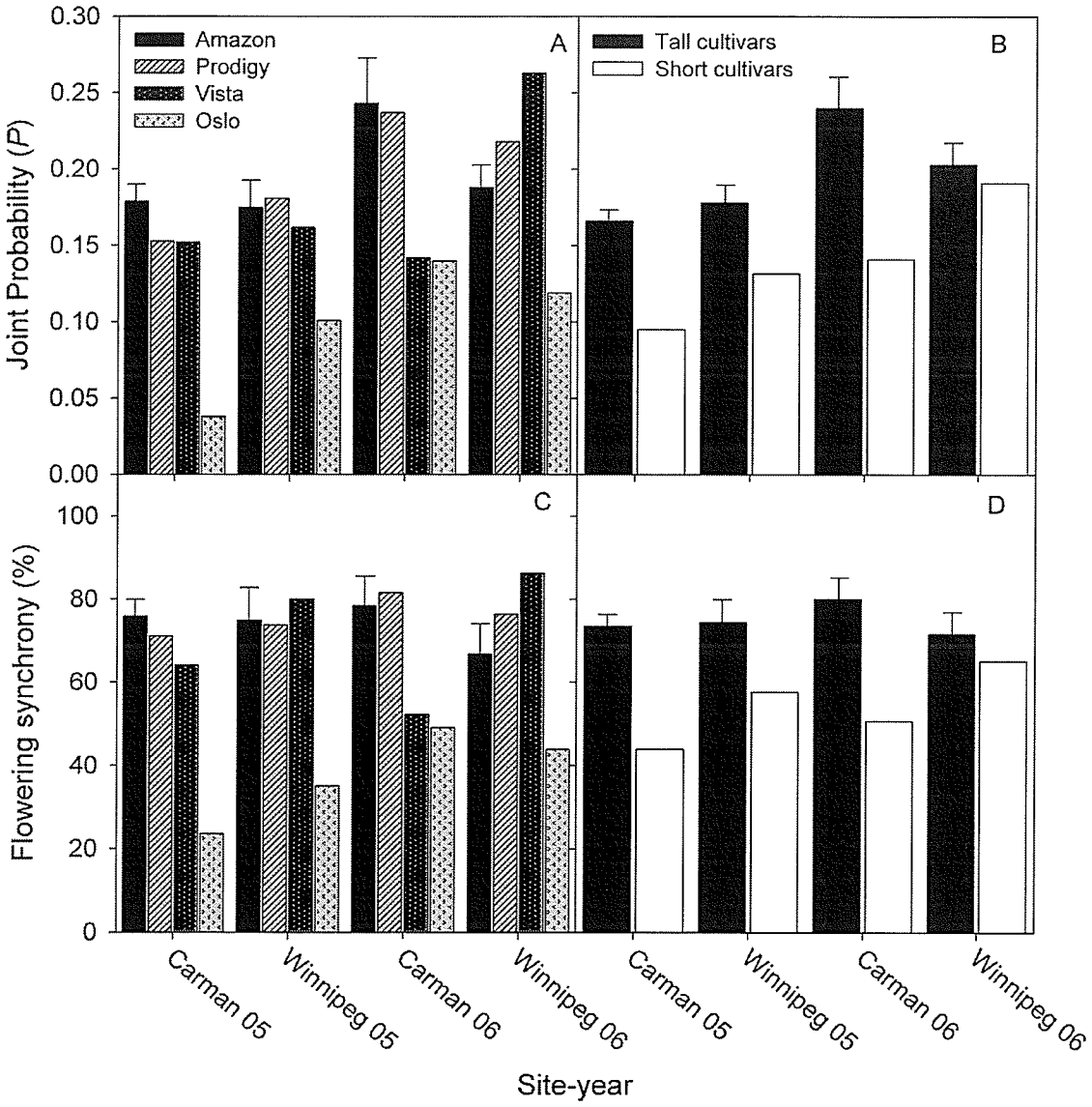

FIGURE 6.4 Relationships between flowering synchrony (A) and the joint probability of synchronous flowering (B) of spring wheat as a function of crop genotype. Mean differences between tall vs. short genotypes for flowering synchrony (C) and joint probability (D) as derived from single degree of freedom contrasts. Field studies were carried out at Carman and Winnipeg, MB, Canada, in 2005 and 2006. Bars indicate ± 1 standard error of the difference between means.

TABLE 6.6 Correlations between flowering synchrony and the probability of synchronous flowering (joint probability) with crop height, the difference between height of the volunteer and the crop, and fertile tiller production (spikes m^{-2}) for spring wheat plants in 2005 and 2006. Correlations in bold font where significant at $P \leq 0.05$.

Site	Year	Height	Height Differential	Crop spikes m^{-2}
		Flowering Synchrony		
Carman	2005	0.90; ***P* <0.001**	0.93; ***P* <0.001**	-0.19; *P*=0.48
Winnipeg	2005	0.58; ***P* <0.05**	0.65; ***P* <0.01**	-0.35; *P*=0.19
Carman	2006	0.69; ***P* <0.01**	0.71; ***P* <0.01**	-0.23; *P*=0.40
Winnipeg	2006	0.28; *P*=0.29	0.19; *P* =0.48	-0.23; *P*=0.40
		Joint Probability		
Carman	2005	0.89; ***P* <0.001**	0.91; ***P* <0.001**	-0.25; *P*=0.34
Winnipeg	2005	0.70; ***P* <0.01**	0.75; ***P* <0.001**	-0.40; *P*=0.13
Carman	2006	0.64; ***P* <0.01**	0.70; ***P* <0.01**	-0.25; *P*=0.35
Winnipeg	2006	0.25; *P*=0.36	0.15; *P*=0.58	-0.20; *P*=0.47

from 7 to 9 d during a cooler flowering period. Although variation among site-years precluded a combined analysis of the data, our results are robust in that we quantified flowering phenology under substantially varied environmental conditions.

Although previous studies indirectly reported differences in flowering duration among wheat genotypes (Bauer et al., 1984; Hucl, 1996; Matus-Cádiz et al., 2007), we have directly quantified variation in flowering phenology among spring wheat genotypes. Our results confirm that despite the dominant effect of environmental conditions on flowering characteristics, genotype has a profound influence on flowering phenology in spring wheat. No differences were observed among genotypes in time from sowing to emergence (data not shown), yet differences were observed among genotypes in time from emergence to anthesis (Table 6.4). This may be due to Oslo possessing an intrinsically higher growth rate than the other genotypes included in this study. Growth rate and phyllochron intervals are known to vary among genotypes (Bauer et al., 1984; Karimi and Siddique, 1991). Genetically, Oslo may be earlier flowering than the other spring wheat genotypes included in this study. The increased flowering period duration in Oslo over that of the other genotypes (Table 6.4) supports this assertion.

Considerable variation was found in the degree of flowering synchrony between cropped and volunteer wheat (Fig. 6.4). This variation was strongly influenced by genotype, with no interactions observed between crop genotype and density. Early flowering of Oslo resulted in a low proportion of its flowering period being synchronous with volunteers (Figs. 6.2 and 6.4). Consequently, the probability of synchronous flowering between Oslo and volunteer wheat was generally low in all site-years (Fig. 6.4A). While little variation in flowering synchrony existed between Amazon and Prodigy among site-years, Vista exhibited considerable variation, suggesting that flowering characteristics in Vista are more responsive to environmental conditions in comparison to the other genotypes (Fig. 6.4C). These results concur with those of Wong and Baker (1986) who reported that accumulated thermal time from sowing to anthesis in western Canadian wheat genotypes ranged considerably, from a low of 543 to a high of 695 GDD.

Interestingly, semi-dwarf genotypes consistently exhibited less flowering synchrony and lower probabilities of synchronous flowering with volunteers than the tall genotypes, a trend that was significant in three of four site-years (Fig. 6.4). Joint probability and flowering synchrony values were often highly correlated with crop height in those three site-years, and the correlations increased in magnitude when correlated with the difference between crop and volunteer height (Table 6.6). These trends were significant ($P < 0.01$) and although these data support sowing short cultivars to reduce flowering synchrony between semi-dwarf wheat crops and taller volunteer wheat populations, we cannot conclusively recommend this for two reasons. First, volunteer flowering phenologies were largely unaffected by crop height (Table 6.5) and therefore, these results may not be due to shading, as was initially hypothesized. On the contrary, differences between tall and short genotypes could have stemmed from a shorter intrinsic flowering period among the tall genotypes, coupled with a fortuitously greater degree of overlap with volunteers (Table 6.4, Fig. 6.2). Second, variation in flowering characteristics reported between genotypes restricts the scope of the conclusions that can be drawn because, for logistical reasons, only a single volunteer genotype was included in this study. Therefore, the relationship between height and flowering synchrony that we observed may have been somewhat specific to the combinations of genotypes used. Further testing is required to discern whether shading by taller genotypes accelerates flowering in shorter wheat genotypes and how this in turn influences flowering phenology.

Nevertheless, while the magnitude of the responses observed will be genotype-specific, this study has clearly indentified differences in both flowering characteristics and synchrony levels between spring wheat genotypes (Fig. 6.4). Our results suggest that with sufficient knowledge of the flowering characteristics of spring wheat genotypes, producers could potentially utilize the variation in flowering phenology that exists among genotypes to limit synchronous flowering with transgenic wheat volunteers. Flowering characteristics of genotypes are not readily accessible at present, but should be easily obtained from yield trials or local plant breeders and could be published in variety selection guides (along with days to maturity). This information could serve as a practical

guide to variety selection in cases where coexistence between transgenic and non-transgenic wheat is pertinent. Flowering asynchrony has been implicated as a major factor preventing hybridization between source and receptor maize (*Zea mays* L.) plots (Halsey et al., 2005). Similarly, Della Porta et al. (2008) demonstrated a 50% reduction in PMGF between source and recipient maize plants whose flowering time differed temporally by 6 d. Because volunteer wheat in our study flowered for only 5 to 6 d, shifts of 4 to 6 d in wheat flowering could reduce flowering synchrony by 75 to 100%, which could have important ramifications for PMGF.

While the results of this study clearly indicate that genotypic and environmental influences on flowering phenology are strong, we cannot dismiss the effects of crop density. Both time of flowering onset and progression through the flowering period were generally earlier and expedited, respectively, at high compared to low crop densities (Fig. 6.3). Moreover, a near-linear decline in volunteer flowering period duration was observed with increasing crop density in three of four site-years, with flowering duration being 19% greater at a target density of 75 compared to 600 plants m^{-2} (Table 6.5). Interestingly, crop density exerted a greater effect on the flowering period of volunteer plants than on crop plants, suggesting competition for limiting resources at high densities resulted in accelerated flowering. Accelerated flowering at higher densities has been previously reported for wheat (Davidson and Chevalier, 1990; Turner et al., 1994) and other crop species (Palmblad, 1968; Schmitt et al., 1987). In Canada, recommended crop densities for spring wheat range from 250 to 300 plants m^{-2} (Manitoba Agriculture, Food, and Rural Initiatives, 2008) and it is important to note that no significant differences between a target density of 300 and 600 plants m^{-2} were observed for either crop or volunteer flowering period duration (Tables 6.4 and 6.5). Moreover, increasing crop density had no effect on flowering synchrony (data not shown) and therefore, our results do not support the premise of increasing crop density to reduce crop and volunteer flowering period duration and synchronicity of flowering. However, ensuring spring wheat crop densities are above 200 plants m^{-2} appears important for reducing the flowering period and potential for hybridization as a result of synchronous flowering between cropped and volunteer wheat.

In summary, this study has provided evidence for variation in flowering synchrony among spring wheat genotypes. This variation was largely attributed to divergence in the flowering phenologies of the four genotypes examined, with flowering onset and progression occurring earlier for Olso than the other genotypes. Tall genotypes consistently exhibited a higher degree of and probability for synchronous flowering than semi-dwarf genotypes. While this study did demonstrate a near-linear decline in volunteer flowering period with increasing crop densities, crop density did not influence flowering synchrony or the probability of synchronous flowering. Despite a short flowering period, considerable potential exists for a high degree of synchronous flowering between cropped and volunteer spring wheat populations. Nevertheless, opportunity exists to reduce flowering synchrony between cropped and volunteer wheat populations by using temporal variation in flowering phenologies as a barrier to flowering synchrony and potential PMGF, provided an adequate crop density has been attained.

7.0 Low crop densities promote pollen-mediated gene flow between cropped and volunteer wheat

7.1 Introduction

The commercialization of transgenic crops is frequently accompanied by a risk assessment of the potential impact of that transgenic crop on the environmental and ecological systems within which it will be cultivated (Chandler and Dunwell, 2008). Of primary focus in risk assessment is the probability of gene flow to non-transgenic plants and wild relatives. Gene flow among cultivars of major field crops traditionally has been a concern only in cultivar development and seed production, with little attention paid to gene flow at the commercial scale (Hanson et al., 2005b). However, the wide-scale cultivation of transgenic or identity-preserved crops with special traits does require higher purity standards, and has resulted in keen interest regarding the issue of crop-to-crop and volunteer-to-crop gene flow (Conner et al., 2003; Kershen, 2004).

Crop-to-crop and crop-to-volunteer gene flow occurs frequently in agricultural crops (Ellstrand, 2003a), and is of concern in any situation where a specified genetic purity is required. Mechanisms of gene flow vary among plant species, but generally consist of either movement of a sporophytic ($2n$) seed from one location to another, and/or the migration of haploid pollen grains via PMGF followed by subsequent hybridization (Slatkin, 1987; Ellstrand, 2003b). The former provides spatial and temporal migration of the transgene (often through the establishment of volunteer populations), whereas spatial migration is facilitated by the latter (Brûlé-Babel et al., 2006). While seed movement is undeniably an important factor in structuring the genetic composition of populations, pollen dispersal is considered the main mode of gene flow in flowering plants because it

provides a direct mechanism for gene flow into the same or related species (Levin and Kerster, 1974). Depending on the nature of the transgenic trait, unintended migration of transgenes into non-transgenic crops, volunteer populations, or wild relatives could alter farming practices, contaminate other crops, or irreversibly alter the ecosystem (Baker and Preston, 2003). Before a transgenic trait is introduced, it is necessary to understand how agronomic factors affect the reproductive biology, flowering synchrony, movement of the transgene, and ultimately, the fate of the transgene in the agroecosystem.

Several field trials have been conducted in North America in anticipation of the eventual release of transgenic wheat (reviewed in Chapter 2). Wheat is considered a self-pollinating species with PMGF rates usually less than 1% (Poehlman and Sleper, 1995). Pollen-mediated gene flow, which is facilitated by wind-borne pollen, is known to vary with the reproductive biology of the species or cultivar, as well as with environmental conditions before and after flowering (Bitzer and Patterson, 1967; De Vries, 1971; Waines and Hegde, 2003). The tendency for PMGF to decline with increasing distance from the pollen source also is well-established for spring wheat (Matus-Cádiz et al., 2004; Hanson et al., 2005b; Matus-Cádiz et al, 2007) and has even been modeled (Gustafson et al., 2005; Gaines et al., 2007a). However, little information is available regarding the influence of agronomic practices on PMGF in spring wheat.

The development of wheat genotypes with novel traits has raised concerns regarding the presence of volunteer wheat populations and the role they may play in facilitating transgene movement (Brûlé-Babel et al., 2006). Because some markets may require segregation between transgenic and non-transgenic varieties, legitimate concern exists surrounding the possible contamination of non-transgenic wheat crops by volunteers, either through seed contamination or PMGF. Volunteer wheat is the 12th most abundant weed in western Canada, persisting on at least 11% of fields at an average density of 6 plants m^{-2}, but infestations as high as 281 plants m^{-2} have been reported (Leeson et al., 2005). Because of the predominantly inbreeding nature of wheat species, in theory genetic purity should be relatively easy to maintain. However, the potential for intraspecific PMGF in wheat can be as high as 10% (Lawrie et al., 2006), and PMGF has been identified as far as 2.75 km from the pollen source (Matus-Cádiz et al., 2007).

Furthermore, off-types are frequently indentified in pedigreed seed production in Canada (Hucl et al., 2004). A similar study in the United States found that approximately one third of the growers whose seed was sampled contained imazamox-resistant (IR) wheat varieties that were adventitiously present (AP) in non-IR wheat samples, with four samples actually exceeding the 0.01% threshold for off-types in certified wheat seed (Gaines et al., 2007b). Thus, volunteer wheat populations represent a significant challenge to meeting and maintaining genetic purity and AP thresholds in non-transgenic wheat crops.

Volunteer wheat can emerge as a single cohort early in the growing season (De Corby et al., 2007) or as multiple cohorts throughout the growing season (Anderson and Soper, 2003; Harker et al., 2005). Emergence timing of volunteer wheat is of concern because seed survival and fecundity of volunteer plants is dependent on emergence timing of volunteers (De Corby et al., 2007). Of greater concern is that emergence timing can also affect flowering synchrony with the crop (Chapter 4) and more importantly, can profoundly influence PMGF (Chapter 5). Volunteer emergence at the same time or shortly after crop emergence resulted in the greatest levels of PMGF (Chapter 5), but these volunteers cannot be selectively controlled in and/or removed from wheat crops. This underscores the conclusions of De Corby et al. (2007) who suggested that "Efforts and attention should be directed toward achieving very high levels of volunteer wheat control."

There are, however, methods which could offset gene flow arising from inadequate control of wheat volunteers. Spring wheat genotypes have demonstrated wide variation in their propensity for gene flow with some genotypes such as cv. Katepwa demonstrating low frequencies of gene flow (0.1%), while others such as cv. Oslo (6.1%) and cv. Glenlea (10.6%) typically exhibit high frequencies of PMGF (Hucl, 1996; Hucl and Matus-Cádiz, 2001; Lawrie et al., 2006). Manipulating the crop canopy by growing semi-dwarf genotypes has been shown to influence flowering synchrony (Chapter 6) and may also have merit in reducing PMGF between volunteer and cropped wheat populations. Martin (1990) documented greater frequencies of PMGF in semi-dwarf genotypes, although plant height was not correlated with PMGF. Still another possibility may be

increasing spring wheat plant population densities to dilute PMGF from volunteers, thus increasing the receptor to source plant ratio. Therefore, the objectives of this study were: 1) to determine the influence of crop height and plant population density on PMGF between volunteer wheat populations and spring wheat crops and 2) to assess the relationship between PMGF, flowering synchrony, and yield components in spring wheat.

7.2 Materials and Methods

7.2.1 Experimental Procedures

Field trials were conducted in 2005 and 2006 at two locations near Winnipeg and Carman, Manitoba, Canada. The soil type at Winnipeg was a Boralfic Haploborol clay (20% sand, 26% silt, 55% clay) while soil at Carman was an Udic Haploboroll, Eigenhof Series sandy loam (46% sand, 24% silt, 30% clay), both typical soils found in the Red River Valley region of Manitoba. Plots at Carman received a tillage pass in the fall and spring (to incorporate fertilizer) with a field cultivator, while the area at Winnipeg was harrowed immediately prior to planting. To avoid the confounding effects of native volunteer wheat populations, 900 g a.e. ha^{-1} glyphosate was applied prior to crop emergence to control emerged weeds. At each location, experimental sites were chosen that had not been planted to wheat for the previous two years. To further isolate the experimental area from competing pollen, the experiments were seeded at least 300 m from any adjacent wheat fields.

Plots were arranged in a split-plot experimental design with four replications at each location. Crop density was the main plot effect and crop cultivar was the subplot effect. Main plots (6- x 17-m) were randomized within replicates and included four spring wheat target crop densities (75, 150, 300, 600 plants m^{-2}). Isolation distances of 10 m between each main plot, 10 m between replicates, and 3 m between sub-plots were implemented to minimize plot-to-plot outcrossing. Seeding rates were adjusted for genotypic differences in thousand kernel weight and germination test results to achieve the desired plant

population densities. Subplots (6- x 2-m) were randomized within main plots and consisted of two tall (cv. Prodigy, Graf et al., 2003; cv. Amazon, Anon., 2005c) and two semi-dwarf (cv. Oslo, Graf et al., 1990; cv. AC Vista, DePauw et al., 1998) spring wheat cultivars. Volunteer (cv. CDC Imagine, IR) spring wheat served as the pollen source and was planted at a constant target density of 30 plants m^{-2} in alternating rows such that each row of crop was bordered on either side by a row of volunteer plants (paired-pollinator rows).

Volunteer and cropped wheat populations were planted at a depth of 2 cm using a low-disturbance, double-disc plot drill with 15.2 cm row spacing. Fertilizer was broadcast prior to seeding based on soil test recommendations. Seeding dates in 2005 and 2006 were 11 May and 9 May at Carman and 16 May and 12 May at Winnipeg, respectively. Weed, disease, and insect (aphid) control were achieved using standard, widely-recommended chemicals as in Chapter 6. A weather station located within 200 m of each experiment measured air temperature, relative humidity and precipitation throughout the flowering period as in Chapter 5.

7.2.2 Data collection

Cropped and volunteer wheat plant population densities were quantified by counting plants in two randomly-selected, non-edge rows (1 m in length) in each plot three to four weeks after crop emergence. Plant height of cropped and volunteer wheat at flowering was determined by measuring the height of six plants per plot. At maturity, a subsample of cropped wheat spikes was harvested by hand from two 1 m rows in each plot. Each subsample was immediately threshed using a Wintersteiger (Wintersteiger Seedmech, Saskatoon, SK) plot combine. Another subsample of cropped wheat spikes was cut by hand from a 1 m row in each plot. Both samples were placed in individually labeled cloth bags and transported to a central processing facility where they were dried to a uniform moisture content. Grain samples were cleaned and weighed, with the total weight of each sample divided by the average weight of one-thousand seeds (TKW) to estimate the total number of seeds in each sample. Cropped wheat TKW was calculated by determining the weight of 500 kernels and multiplying by two. Each 1 m row sample of wheat spikes was

counted to determine the number of fertile tillers (spikes). In addition, five spikes per sample were threshed by hand and the number of seeds per spike recorded.

Because copper (Cu) deficiency has been reported to cause male sterility in wheat plants (Graham, 1975), Cu characterization of soil and seeds was conducted at each site in both years. Soil Cu characterization consisted of taking a composite soil sample of six subsamples (0- to 15-cm depth) in a W-shaped pattern across the length of each site. Each composite soil sample was ground to pass through a 2 mm plastic sieve and analyzed for extractable Cu by DTPA extraction (Lindsay and Norvell, 1978). Seed Cu characterization was conducted on a composite seed sample of four subsamples (one across each block) for two cultivars (Amazon and AC Vista) at each site in both years. Samples were ashed in a muffle furnace, digested with nitric acid (Kaira, 1998), and analyzed with inductively coupled plasma mass spectrometry (USEPA, 1994). Copper deficiency and the resulting male sterility can also lead to increased ergot infection (Bailey et al., 2003) and therefore, these samples were also screened for the presence of ergot (*Claviceps purpurea* Fr.:Fr. Tul.) bodies by counting the number of ergot-infected kernels in each sample. Ergot-infected kernels were expressed as a percentage of the number of kernels in the sample by dividing the number of ergot infected kernels by the estimated total number of seeds in each sample (calculated based on TKW).

7.2.3 Sample Screening

To determine the extent of PMGF, a subsample (approx. 2000 seeds) of seed from each grain sample (one per plot) was sown in the field the following spring (2006 and 2007). To ensure the method would be able to select both homozygote and heterozygote individuals, four control plots were also established for the resistant (R) genotype (CDC Imagine) and all four susceptible (S) genotypes (one for each genotype in each block). At the 2- to 3- leaf stage, a foliar application of 20 g a.i. ha^{-1} imazamox (BASF Canada Inc., Toronto, ON) was applied with a plot sprayer calibrated to deliver 56 L ha^{-1} spray volume at 275 kPa using 11001 Teejet nozzles (Spraying Systems Co., Wheaton, IL), with Merge® surfactant (BASF Canada Inc., Toronto, ON) added at 0.5% v/v to the spray solution. Approximately 16 d after imazamox application, plants that survived the

herbicide application were counted in each plot. Surviving plants that exhibited an injured (coleoptilar tillering) phenotype would most likely be heterozygous and resulted from hybridization between the test cultivar and the herbicide tolerant CDC Imagine (Pozniak and Hucl, 2004).

To eliminate any potential homozygous R contaminants and S escapes that may have been present in the samples, a second herbicide screening was used to confirm hybridization. In each F_1 plot, all plants (where logistically possible) surviving imazamox application (putative F_1 hybrids) were grown to maturity and subsequently harvested by hand. The resulting F_2 progeny were then screened under controlled environment conditions to confirm that they were from F_1 plants. Sixteen seeds of each surviving F_2 progeny were sown in 27 x 54 cm flats containing 48 cells, with two seeds sown per cell. Flats were placed in a growth room with a 16-h photoperiod, 21/15°C day/night temperature, and an approximate radiance of 480 μmol m^{-2} s^{-1}. At the two leaf stage, imazamox was applied at 12 g a.i. ha^{-1} using a cabinet sprayer equipped with a flat fan nozzle (TeeJet 80015 nozzle tip) calibrated to deliver 117 L ha^{-1} of spray solution at 310 kPa in a single pass over the foliage, with Merge® added at 0.5% (v/v) to the spray solution. In Chapter 3 we demonstrated that the optimal dose of imazamox for growth room discrimination of R, S, and heterozygous intermediate (I) plants is 12 g a.i. ha^{-1}. The hybrid nature of seeds was confirmed by visually identifying families that segregated in the 3:1 alive:dead ratio (same symptoms as above) expected for this single gene, semi-dominant trait (Pozniak and Hucl, 2004) in the $F_{1:2}$ generation.

A total of ca. 14,880 $F_{1:2}$ seedlings were screened from the 930 F_1 plants harvested across the four location-year combinations. Due to the semi-dominant expression of the trait controlling imazamox-resistance in spring wheat (Pozniak and Hucl, 2004), individual plant variability could conceivably result in misclassification of F_1 plants in the screening procedure. The probability of not detecting a segregating F_1 plant in the $F_{1:2}$ generation is determined by population size (Rédei, 1982) such that:

$$n = \log(1 - P)/(\log(u) - \log(u + 1)) \qquad (7.1)$$

where n is the population size, P is the probability of obtaining at least one S seedling, and u is the proportion of the surviving homozygous (R) and heterozygous (I) seedlings

relative to the S seedlings. Thus, using the above criteria and screening 16 seedlings in the $F_{1:2}$ generation for each individual surviving F_1 plant, the possibility of misclassifying a genotypically segregating F_1 plant as a homozygous R or S plant was approx. 1 in 100, which is relatively low.

7.2.4 Statistical Analysis

Prior to analysis, residuals for all data were tested for normality and homogeneity of error variance with the UNIVARIATE procedure in SAS (SAS Institute, 2004). Data for spikes plant^{-1} were log (base 10) transformed to normalize the residuals and in this case, ANOVA was performed on the transformed data. Crop yield and yield component data were subjected to a two-way (crop density x genotype) ANOVA using the mixed-model procedure of SAS (Littell et al., 1996), with degrees of freedom calculated using Satterthwaite's approximation method. Fixed effects and random variance components were estimated by restricted maximum likelihood. Crop genotype, density, and their interaction were considered fixed effects whereas blocks, locations, years, and their interactions with fixed effects were considered random effects. Within the mixed procedure, a Wald *Z* statistic was employed to test the significance of the random effects of block, location, and year and their interaction with crop genotype and density. This tests the significance of covariance parameter estimates and indicated whether or not data could be pooled across random effects. ESTIMATE statements were used to make comparisons among crop densities, while Tukey's honestly significant difference (Tukey, 1953) was used to compare genotype means with treatment effects declared significant at $P < 0.05$.

To determine whether crop genotype and density affected PMGF frequencies, data from each plot were expressed as the percentage of confirmed heterozygotes in a sample (plot) using the following equation:

$$\text{PMGF (\%)} = (\text{number of confirmed heterozygotes in a sample} / \text{total number of seeds in a sample}) \times 100 \quad (7.2)$$

Nonlinear regression analysis was employed to estimate PMGF frequency as a function of crop density in the four genotypes examined (PROC NLIN, SAS Inst., 2004). For each crop genotype, the following exponential decay function was fit to PMGF data:

$$P_{GF} = a \times e^{[-i(D)]} \tag{7.3}$$

where P_{GF} is the percentage PMGF, D is actual observed crop density in each plot (plants m^{-2}), and a and i are the fitted regression parameters. Parameter a is the maximum estimated PMGF at $D = 0$ and i is the frequency at which PMGF declines with increasing D. Equation 7.3 was fit to the data separately for each genotype, location, and year. The Gauss-Newton iterative procedure was used to find the values of the model parameters that minimized the sums of the squared deviations between observed and fitted values. Lack of fit tests could not be performed because plant population densities were unique for each plot and consequently, lacked the replication necessary to provide an estimation of pure error. Therefore, the goodness-of-fit of each regression model to each individual data set was evaluated using an approximate F statistic and a *Pseudo* R^2 (Schabenberger and Pierce, 2002). Differences in parameter estimates between genotypes, locations, and years, were compared using the extra sum of squares (ESS) principle for nonlinear regression as described by Ratkowsky (1983). Where parameters were determined to differ significantly between genotypes, locations, or years, 95% confidence intervals around the parameter estimates were used to determine which data sets differed in their values of each model parameter (Jasieniuk et al., 1999).

Correlation analysis was used to examine the relationships between height, yield components, flowering synchrony and joint probability data from Chapter 6, and PMGF. Where residuals were normally distributed, Pearson correlations were used whereas non-parametric Spearman Rank correlations were employed where residuals were non-normal and could not be transformed. Because we were primarily interested in correlating PMGF with the other variables, and because PMGF data could not be combined across locations, separate correlations were conducted for each location with data pooled across years.

7.3 Results

Weather patterns during the pollination period varied substantially between years and locations (Table 7.1). Average daytime temperature during the flowering period ranged from 21.5 to 25.5°C at the two experimental sites. Mean daily relative humidity during anthesis was lowest at Carman in 2006 (53.2%) and highest at Carman in 2005 (79.6%). Precipitation during the flowering period also exhibited variation between locations and years, ranging from 0.0 mm at Winnipeg in 2006 to 81.8 mm at Winnipeg in 2005. Likewise, precipitation during the flowering period was nine-fold higher at Carman in 2005 than in 2006. Duration of flowering ranged between 7 to 9 d and was a day shorter at both sites in 2006 as a result of higher temperatures.

Due to significant year and location effects, grain yield and yield components data were analyzed within site-year combinations (data not shown). The main effect of genotype was significant ($P \leq 0.05$) for spring wheat grain yield and seeds spike^{-1} in nearly all site-years (Table 7.2). Grain yield both among and within genotypes varied considerably between site-years, ranging almost three-fold from 1911 kg ha^{-1} for Oslo at Winnipeg in 2006 to 5400 kg ha^{-1} for AC Vista at Carman in 2005 (Table 7.3). In three of four site-years, grain yield for Oslo was less than for all other genotypes. The number of seeds spike^{-1}, on the other hand, was consistently highest for Prodigy, irrespective of crop density or site-year. Averaged across site-years, 32 seeds were borne on each spike of Prodigy compared with only 30 seeds spike^{-1} in Amazon and 27 seed spike^{-1} in AC Vista and Oslo.

Likewise, the main effect for crop density also was significant ($P \leq 0.05$) for grain yield in three site-years, seeds spike^{-1} at both sites in 2005, and spikes plant^{-1} in all site years (Table 7.2). Grain yield generally increased hyperbolically with increasing density and was least at the lowest crop density (Table 7.3). In contrast, the number of spikes plant^{-1} decreased hyperbolically with increasing density in all site-years. Compared with grain yield at 75 plants m^{-2}, yields (across site-years) were 44% and 43% higher at 300 and 600 plants m^{-2}, respectively. Similarly, wheat plants produced 56% and 72% fewer spikes

TABLE 7.1 Summary of daytime weather during pollination experiments in Carman and Winnipeg, MB, Canada.

Site	Year	Pollination duration	Mean temperature†	Mean relative humidity†	Flowering period precipitation
		d	°C	%	mm
Carman	2005	9	21.5	79.6	38.0
Winnipeg	2005	8	22.2	64.8	81.8
Carman	2006	8	25.5	53.2	4.2
Winnipeg	2006	7	23.0	72.9	0.0

† Values are the averages of data recorded at hourly intervals between 0600 to 1800 h, the period during which pollen shed is greatest in wheat (De Vries, 1972).

TABLE 7.2 Significance (*P*) of the effect of crop genotype, density, and their interaction on crop yield components within each site-year combination.

Site-year	Source	df	Grain Yield	Spikes plant^{-1}	Seeds spike^{-1}
			P value		
Carman 2005	Genotype (G)	3	**0.000**	0.239	**0.009**
	Density (D)	3	**0.041**	**0.000**	**0.015**
	G X D	9	0.610	0.470	0.902
Winnipeg 2005	Genotype (G)	3	**0.001**	0.153	0.163
	Density (D)	3	0.647	**0.001**	**0.001**
	G X D	9	0.328	0.961	0.299
Carman 2006	Genotype (G)	3	**0.000**	0.104	**0.001**
	Density (D)	3	**0.000**	**0.000**	0.053
	G X D	9	0.977	0.396	0.236
Winnipeg 2006	Genotype (G)	3	**0.000**	0.428	**0.000**
	Density (D)	3	**0.004**	**0.000**	0.068
	G X D	9	0.627	0.387	0.323

TABLE 7.3 Crop genotype and density effects on crop yield components within each site-year combination.

	Genotype				Crop target density (plants m^{-2})			
Site-year†	Amazon	Oslo	Prodigy	Vista	75	150	300	600
	Yield (kg ha^{-1})‡							
CAR 05	4944 b	2582 c	4532 b	5400 a	3641 b	4612 a	4738 a	4467 a
WPG 05	2219 b	2529 b	3044 a	3068 a	2484 a	2705 a	2976 a	2694 a
CAR 06	4094 a	2701 c	3135 b	3910 a	2251 d	3056 c	4038 c	4496 a
WPG 06	2567 a	1911 c	2238 b	2287 b	1609 b	2162 a	2624 a	2608 a
	Spikes (log no. $plant^{-1}$) ‡§							
CAR 05	0.155 (1.4)	0.201 (1.6)	0.246 (1.8)	0.198 (1.6)	0.555 a (3.6)	0.319 b (2.1)	0.104 c (1.3)	-0.178 d (0.7)
WPG 05	0.245 (1.8)	0.346 (2.2)	0.323 (2.1)	0.203 (1.6)	0.543 a (3.5)	0.384 ab (2.4)	0.184 bc (1.5)	0.006 c (1.0)
CAR 06	0.247 (1.8)	0.200 (1.6)	0.295 (2.0)	0.267 (1.8)	0.474 a (3.0)	0.339 a (2.2)	0.187 b (1.5)	0.010 c (1.0)
WPG 06	0.347 (2.2)	0.364 (2.3)	0.396 (2.5)	0.327 (2.1)	0.643 a (4.4)	0.448 b (2.8)	0.278 c (1.9)	0.064 b (1.2)
	Seeds (no. $spike^{-1}$) ‡							
CAR 05	27.0 ab	24.2 b	29.3 a	25.2 b	29.6 a	27.4 ab	24.8 bc	23.9 c
WPG 05	27.0 ab	26.1 ab	27.7 a	24.6 b	29.2 a	26.8 a	26.6 a	22.8 b
CAR 06	33.2 b	31.8 b	37.5 a	32.7 a	36.9	35.5	33.2	29.5
WPG 06	32.1 a	27.0 b	33.1 a	23.5 c	32.2	29.3	27.6	26.7

† CAR 05 & 06, Carman 2005 and 2006; WPG 05 & 06, Winnipeg 2005 and 2006.

‡ Means within the same crop genotype or density row followed by the same lowercase letters are not significantly different ($P \leq 0.05$).

§ Data are log (base 10) transformed. Values in parentheses represent means back-transformed to approximate their original value.

plant^{-1} at target crop densities of 300 and 600 plants m^{-2}, respectively, compared with 75 plants m^{-2}. Not only did plants produce progressively fewer spikes plant^{-1} at higher crop densities, they also produced 25% fewer seeds spike^{-1} in 2005 ($P \leq 0.05$). However, crop density did not significantly affect the number of seeds produced per spike at either location in 2006.

Individual field screening plots averaged 1925 plants per plot over 240 plots with approx. 393,410 plants screened for hybridization across two locations over two years and four genotypes (data not shown). Of the 240 samples examined, many had 0% gene flow with only 75 (31%) confirmed as having IR hybrids present; those containing at least one hybrid seed always had less than 0.55% IR plants (Fig. 7.1). Average PMGF (across years) in plots where hybridization was detected was 0.09% at Winnipeg and 0.17% at Carman. Chi-square analysis indicated that segregation ratios of the suspected hybrids fit the expected 3:1 ratio for a single, semi-dominant gene in the $F_{1:2}$ generation at both sites in 2005 and 2006 (Appendix 1). Figure 7.1 summarizes the variation in gene flow data across both sites in 2005 and 2006. Percent PMGF varied among locations and years, with the greatest frequencies of PMGF observed at Carman in 2006 (Fig. 7.1). Mean PMGF (including plots without hybridization) across the entire experiment ranged from a high of 0.08% to a low of 0.02%, and was two- to four-fold higher at Carman than at Winnipeg in both years (Table 7.4).

The exponential decay model (Equation 7.3) used in the analysis provided a satisfactory description of all PMGF data sets as indicated by the significance of approximate *F* tests, as well as R^2 values that ranged from 0.62 to 0.79 (Table 7.5). Model parameters were well-estimated as seen by the relatively small standard errors associated with parameter values (Table 7.5). Standard errors that are less than half of the numerical value of the parameter estimate indicate good estimation (Koutsoyiannis, 1973). Plots of residuals indicated that errors generally conformed to the underlying regression assumptions (data not shown). Although Equation 7.3 was initially fit to each genotype individually, ESS *F* tests indicated that parameter estimates did not differ significantly ($P \leq 0.05$) across genotypes and therefore, a single curve was fit to PMGF data for all genotypes

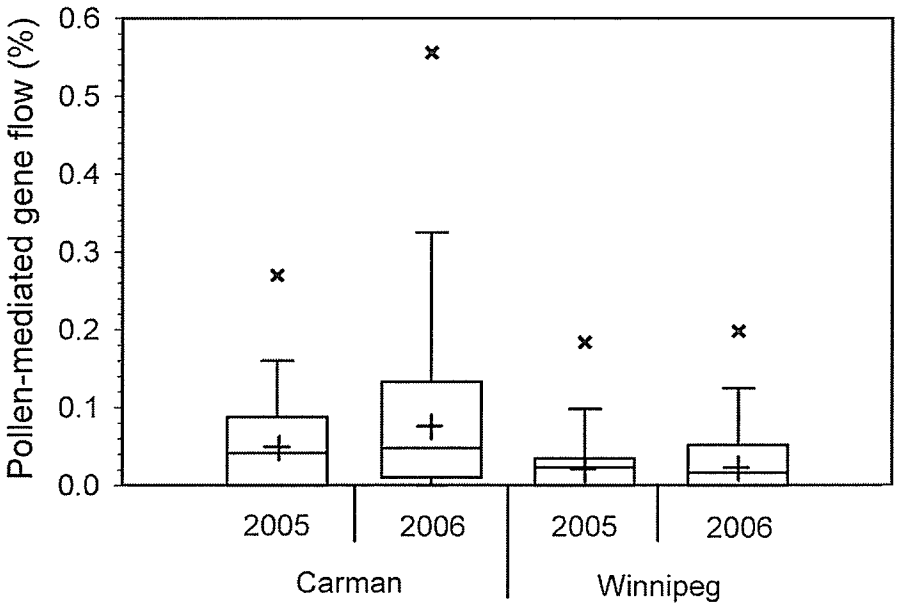

FIGURE 7.1 Box-plot summary of intraspecific pollen-mediated gene flow data between cropped and volunteer spring wheat for four location-by-year combinations (sites) in Manitoba, Canada, in 2005 and 2006. The box is bound by the first and third quartiles with "whiskers" showing the extent of the remaining data beyond the bounds of the box. Outliers are data that occur beyond the ends of the whiskers and are represented symbolically by **x**. The horizontal line within each box depicts the median (50^{th} percentile) while the **+** represents the mean.

TABLE 7.4 Levels of copper in soil and harvested kernels, percentage ergot-infected kernels, and percentage gene flow at Carman and Winnipeg, MB, Canada in 2005 and 2006. Standard errors are presented in parentheses where variation in the response was observed.

Site	Year	Soil test Cu†	Kernel test Cu	Ergot-infected kernels	Mean PMGF‡
		ppm	ppm	%	%
Carman	2005	0.63	1.20 (0.60)	0.631 (0.494)	0.049 (0.012)
Winnipeg	2005	1.73	3.05 (0.05)	0.0	0.023 (0.007)
Carman	2006	0.55	2.30 (0.30)	0.147 (0.021)	0.076 (0.015)
Winnipeg	2006	1.94	3.60 (0.80)	0.0	0.025 (0.006)

† Composite samples were taken at each site and therefore lacked the required variation for a standard error.
‡PMGF, pollen-mediated gene flow was averaged over blocks and treatments, including those where no PMGF was detected.

TABLE 7.5 Model parameter estimates, standard errors, and *pseudo* R^2 values for predicted gene flow from IR wheat (CDC Imagine) to four spring wheat genotypes as a function of crop density.

		Parameter estimate†			
Site	Year	*a*‡	*i*‡	*P* value§	*pseudo* R^2
Carman	2005-2006	0.308 a (0.076)	0.011 a (0.003)	0.000	0.62
Winnipeg	2005-2006	0.136 b (0.026)	0.018 a (0.003)	0.000	0.79
Pooled Model		0.139 (0.031)	0.009 (0.003)	0.000	0.33

† The regression parameters *a* and *i* are the intercept and the rate of exponential decay, respectively.
‡ Means within the same column followed by the same lower case letter are not significantly different ($P < 0.05$) as determined by 95% confidence intervals around each mean.
§ *P* value for significance of approximate model *F* test.

(Table 7.6). Systematic statistical testing with ESS also indicated that parameter estimates did not vary significantly ($P \leq 0.05$) among years, but did vary significantly ($P \leq 0.05$) among locations (Table 7.6). Therefore, regression analysis was conducted individually within each location on data that were pooled across years, cultivars, and blocks.

Crop density had a profound influence on PMGF (Fig. 7.2). Maximum predicted gene flow at Carman, MB, was 0.31% at very low crop densities and declined exponentially to a low of 0.0003% at the highest crop density (600 plants m^{-2}) (Table 7.5, Fig. 7.2). Although maximum estimated gene flow at Winnipeg, MB, was only 0.14%, gene flow at this site also declined exponentially with increasing crop density to a low of 0.0002% at 375 plants m^{-2} (Table 7.5). In fact, 95% confidence intervals around the *i* parameter estimate indicated that it did not vary significantly among locations, demonstrating that the decline in PMGF per unit increase in crop density remained constant between locations (Tables 7.5 and 7.6). In contrast, parameter *a* differed significantly ($P \leq 0.05$) among locations, which suggests that maximum gene flow differed substantially between locations. Indeed, maximum predicted PMGF was two-fold higher at Carman than at Winnipeg.

Not only did PMGF vary greatly between locations (Fig. 7.2, Table 7.4), DTPA-extractable soil Cu levels also were three- to four-fold higher at Winnipeg than at Carman (Table 7.4). Moreover, substantial differences in plant-absorbed Cu were detected in harvested seed, with *t*-tests revealing significant differences (df=6; $P \leq 0.05$) between locations but not between years (df=6; $P = 0.33$) or genotypes (df=6; $P = 0.51$). Mean Cu concentration in seed from Carman (averaged across years) was 1.75 ppm compared to 3.33 ppm in seed from the Winnipeg site. Similarly, the percentage of ergot-infected kernels also was substantially higher at Carman than Winnipeg (Table 7.4). In fact, no ergot-infected kernels were found in subsamples from Winnipeg in either year.

TABLE 7.6 Variation in the exponential function describing cropped wheat PMGF between cropped and volunteer spring wheat among genotypes, sites, and years.

Year	Site	Comparison made	H_o = *a* and *i* do not vary			H_o = *a* does not vary			H_o = *i* does not vary		
			df_1†	df_2‡	F_{calc}§	df_1	df_2	F_{calc}	df_1	df_2	F_{calc}
2005	Carman	Across genotypes	6	8	0.68 NS¶	—	—	—	—	—	—
2005	Winnipeg	Across genotypes	6	8	1.51 NS	—	—	—	—	—	—
2006	Carman	Across genotypes	6	8	2.17 NS	—	—	—	—	—	—
2006	Winnipeg	Across genotypes	6	8	3.05 NS	—	—	—	—	—	—
—	Carman	Across years	2	28	0.33 NS	—	—	—	—	—	—
—	Winnipeg	Across years	2	28	3.11 NS	—	—	—	—	—	—
2005	—	Across sites	2	28	48.50***	2	60	9.71***	2	60	1.99 NS
2006	—	Across sites	2	38	12.88***	2	60	9.71***	2	60	1.99 NS

†df_1, numerator degrees of freedom.
‡df_2, denominator degrees of freedom.
§ F_{calc}, calculated *F* statistic.
¶NS, not significant.

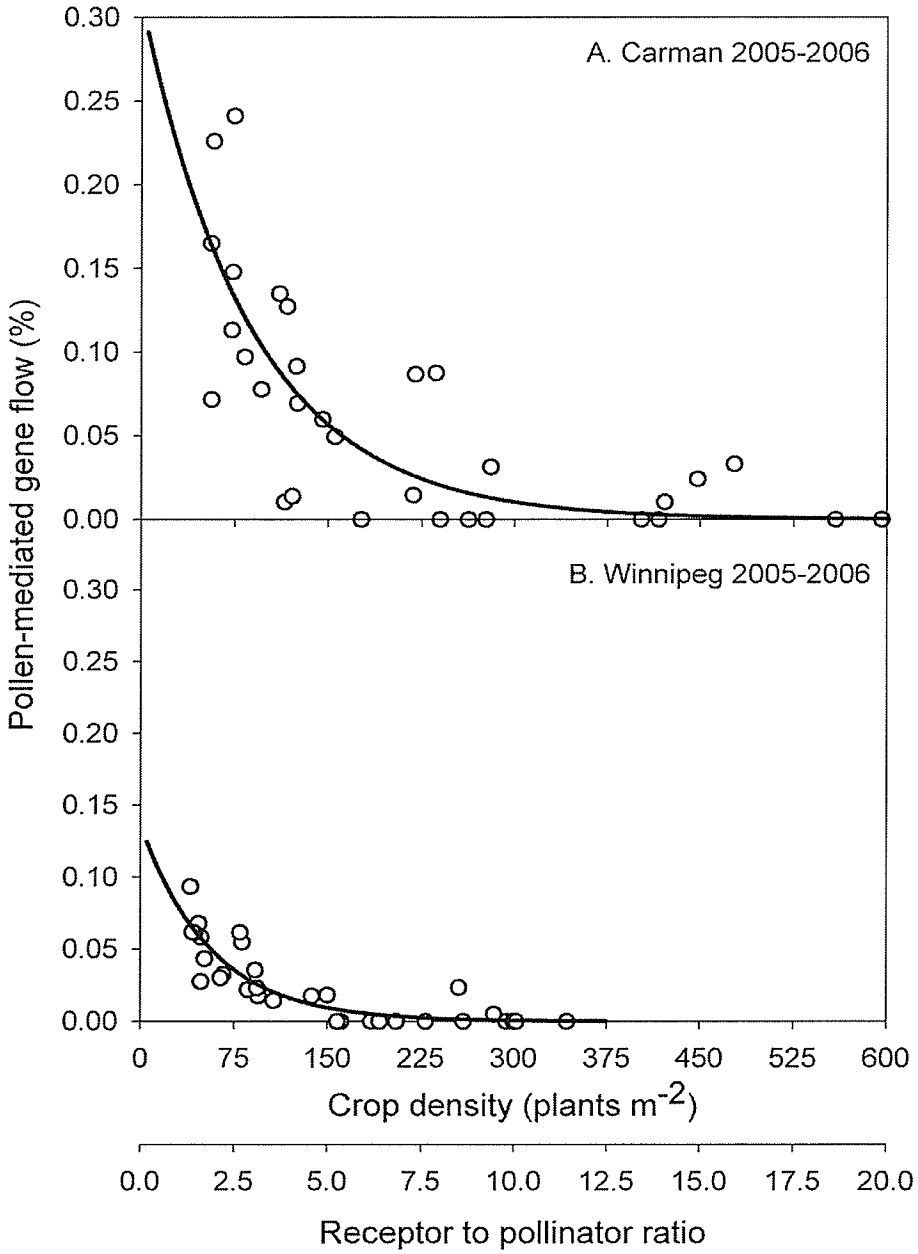

FIGURE 7.2 Mean percent gene flow (and estimated regression curves) from imazamox-resistant spring wheat to four spring wheat genotypes as a function of crop density near (A) Carman and (B) Winnipeg, MB, in 2005-2006. Data were averaged across blocks and a single curve fit to all genotypes within each site across both years, as dictated by extra sum of squares *F* tests. The spread of the predicted regression line within each site encompasses the range of actual observed plant population densities of the spring wheat crop. Parameter estimates are shown in Table 7.5.

Spring wheat crop height was highly correlated (Pearson Correlations = 0.42 to 0.75; $P \leq 0.05$ to < 0.001) with both flowering synchrony and joint probability data from Chapter 6 at both sites (Table 7.7). Tall plants consistently exhibited greater synchronicity of flowering with volunteer wheat plants and therefore also have a higher probability of flowering synchronously with volunteers (Chapter 6). Of the yield components examined, flowering synchrony and joint probabilities were only correlated with seeds spike^{-1} ($P \leq$ 0.05 to 0.001). Although these correlations were moderate to strong, they were not evident for spring wheat grown at Winnipeg. Because flowering synchrony and joint probability were computed from the same data, the high correlation (Pearson Correlations = 0.86 to 0.89; $P < 0.001$) observed between these variables was expected.

Pollen-mediated gene flow in spring wheat showed tremendous association with grain yield and all yield components examined (Table 7.7). Pearson correlations showed a strong positive association (0.66 to 0.79) between PMGF and spikes plant^{-1} that was highly significant ($P < 0.001$) at both sites. Similarly, a more moderate but significant association was detected between gene flow and seeds spike^{-1} (Spearman Rank Correlations = 0.35 to 0.44; $P \leq 0.05$). Taken together, these correlations suggest a strong association between gene flow and crop yield components, whereby more potential fruiting sites via increased seeds spike^{-1} or spikes plant^{-1} supports greater levels of PMGF. We then would also expect grain yield (totality of yield components) to show a strong positive association with gene flow. However, gene flow was consistently negatively correlated (Pearson Correlations = -0.50 to -0.59) with spring wheat grain yield. This can be attributed to spikes plant^{-1}, seeds spike^{-1}, and PMGF being greatest at low crop densities, whereas yield was typically greatest at high crop densities (Table 7.3, Fig. 7.2). Indeed, both spikes plant^{-1} (Pearson Correlations = -0.43 to -0.54; $P < 0.05$) and seeds spike^{-1} (Spearman Rank Correlations = -0.14 to -0.46; $P = 0.44$ to < 0.01) exhibited negative correlations with crop yield at both sites (data not shown). Surprisingly, a weak positive association was detected between PMGF and both flowering synchrony and joint probability, but it was not significant at either location. Likewise, PMGF was not associated with crop height at Carman or Winnipeg.

TABLE 7.7 Within-site correlations between flowering synchrony, joint probability, gene flow, and several response variables for spring wheat plants in 2005 and 2006.

Variable†	Site	Flowering Synchrony‡	Joint Probability‡	Gene flow‡
FS	Carman	-	-	-
	Winnipeg	-	-	-
JP	Carman	0.89***	-	-
	Winnipeg	0.86***	-	-
GF	Carman	0.20	0.30	-
	Winnipeg	0.17	0.12	-
Height	Carman	0.75***	0.80***	0.02
	Winnipeg	0.42*	0.50**	-0.10
Spikes plant^{-1}	Carman	0.23	0.25	0.66***
	Winnipeg	0.07	0.18	0.79***
Seeds spike^{-1}	Carman	0.44*	0.65***	0.44*
	Winnipeg	-0.03	0.18	0.35*
Yield	Carman	0.29	0.09	-0.50**
	Winnipeg	0.02	-0.17	0.59***

†GF, gene flow; FS, flowering synchrony; JP, joint probability.
‡*,**,*** Correlations significant at the 0.05, 0.01, and 0.001 probability levels, respectively. Values given are Pearson correlations except those in bold type, which are Spearman Rank correlations.

7.4 Discussion

Pollen-mediated gene flow between cropped and volunteer wheat populations decreased considerably with increasing crop density, regardless of genotype (Fig. 7.2). Because the density of the volunteer population was held constant in this study, increasing crop density acts to increase the ratio of receptor plants to donor plants. The results of this study demonstrate higher frequencies of PMGF when this ratio is low, with as much as a 400% reduction in gene flow as this ratio increased ten-fold. Although studies examining the role of crop density in the agroecosystem do not exist, these findings are congruent with those conducted in natural systems. Watkins and Levin (1990) reported that the frequency of outcrossing in annual phlox (*Phlox drummondii* Hook.) was negatively correlated with plant density. Similarly, outcrossing rates in common sunflower (*Helianthus annuus* L.) declined two-fold as density increased from 70 to 47 000 plants per hectare (Ellstrand et al., 1978). Morgante et al. (1991) have even suggested that stand density plays a major role in determining outcrossing frequencies in natural plant populations.

Although the results of this study suggest that increasing crop density appears to have merit in reducing PMGF between volunteer and cropped spring wheat populations, it is important to note that recommended planting densities for spring wheat in western Canada are 250 to 300 plants m^{-2} (Manitoba Agriculture, Food, and Rural Initiatives, 2008). Figure 7.2 indicates that reductions in gene flow above crop densities of 300 plants m^{-2} were minimal and thus it would not be warranted, on a practical basis, to increase crop densities to minimize PMGF. However, our results do demonstrate the importance of achieving a minimum crop density of 175 to 200 plants m^{-2} given that below these densities, PMGF increases exponentially. Achieving minimum crop densities will be important especially for non-transgenic wheat producers and organic farmers who must prevent genetic admixture in their wheat crops.

Gene flow and the level of contamination in these experiments was congruent with that expected based on previous reports of gene flow in spring wheat (Table 7.4, Fig. 7.2), which range between 0.1% to 10.6% (Hucl and Matus-Cádiz, 2001; Matus-Cádiz et al.,

2004; Lawrie et al., 2006). Genotypes were chosen for this study because they varied in height, genetic background, and were reported to have similar maturity with volunteer plants so as to maintain some level of flowering overlap. Lawrie et al. (2006) characterized the propensity for Prodigy to outcross as intermediate, while Oslo has demonstrated high levels of outcrossing in other studies (Hucl and Matus-Cádiz, 2001; Lawrie et al., 2006). The propensity of Amazon and AC Vista to outcross has not been determined, although cv. Glenlea, the recurrent parent of Amazon, demonstrated high levels of outcrossing in previous studies (Hucl, 1996; Lawrie et al., 2006). Based on the results of this study, which failed to detect differences in PMGF between genotypes, Amazon and AC Vista appear to be similar to Oslo and Prodigy in regards to their propensity for PMGF (Tables 7.5 and 7.6; Fig. 7.2).

The lack of genotypic variation in PMGF observed in our study (Table 7.6) contrasts with previous findings in spring wheat (Martin, 1990; Hucl, 1996; Lawrie et al., 2006). However, there are two plausible reasons for this. First, Oslo is known to be a high outcrossing genotype but as reported in Chapter 6, its flowering period overlapped only briefly with the volunteer (2 to 3 d). Although Hucl (1996) documented a twenty-fold range in outcrossing rates among 10 Canadian spring wheat cultivars (where outcrossing in Oslo was 4.6%), the author used three pollinator seeding dates to maximize flowering synchrony with receptor plants. A similar range in PMGF was reported for Kansas hard red winter wheat cultivars in one year where outcrossing was positively correlated with flowering duration; however, maximum variation between cultivars was only ten-fold and three-fold in years where no correlation was detected (Martin, 1990). Second, the effects of increasing plant density on PMGF were so strong that genotypic effects may have been overridden by those of density, likely resulting in greater variation within rather than among genotypes. In either case, the lack of differences in PMGF observed in the current study appears to be the exception rather than the rule (Martin, 1990; Hucl, 1996; Lawrie et al., 2006).

Height of the pollen source can affect the magnitude of pollen fluxes in plant canopies (Aylor, 2005). However, variation in PMGF in this study could not be attributed to differences in plant height (Table 7.6, Fig. 7.2). While attempts were made to fit

regression curves to the semi-dwarf and tall genotypes separately, ESS tests indicated that no significant differences existed between the two with regard to PMGF (Table 7.6). This is the second study to report no association between crop height and PMGF in wheat. Martin (1990) also found no significant relationship between crop height and outcrossing among 12 hard red winter wheat genotypes, although semi-dwarf cultivars did have higher outcrossing rates than taller cultivars. Taken together, the results of these studies suggest that despite the reported "heavy" nature of wheat pollen compared with other grass (*Poaceae*) pollen (Lelley, 1966; cited in Waines and Hegde, 2003), it appears only a small quantity of pollen falls directly downward. Should the majority of pollen move downward, we would have expected to see significantly greater PMGF in semi-dwarf genotypes. Although vertical pollen flux has not been investigated in wheat, the vertical flux of corn pollen was observed to decrease steadily with depth into the canopy (Aylor, 2005). Jarosz et al. (2005) reported that corn pollen concentrations close to the source plot were a direct function of source strength. Source strength in the current study varied with crop density and the strong density effects observed in this study may have overridden those for height. Nevertheless, previous observations of PMGF in spring wheat at distances of 200 m and 2750 m from the pollen source by Matus-Cádiz et al. (2004) and Matus-Cádiz et al. (2007), respectively, provide strong support for the vertical movement and transport of wheat pollen grains.

Gene flow in spring wheat is known to depend on such environmental factors as ambient temperature, moisture, and precipitation (Waines and Hegde, 2003). These factors collectively influence the degree of floret opening of the flowers, duration of stigma receptivity, and pollen viability (De Vries, 1971). Despite two- to three-fold greater variation among years than sites with respect to environmental conditions (Table 7.1), statistical tests indicated PMGF data in the current study could be combined across years but not across locations (Table 7.6). Mean gene flow rates at Carman were two-fold and three-fold greater than at Winnipeg in 2005 and 2006, respectively (Table 7.4). Taken together, this indicates that edaphic factors specific to each location influenced PMGF to a greater degree than environmental conditions. To our knowledge, this is the

first study in spring wheat to actually test statistically whether PMGF data could be combined among locations, years, or both.

Considerable variation in DTPA-extractable soil Cu levels was observed between locations in the current study, which in both years, led to significant differences between Cu levels in harvested seed (Table 7.4). Moreover, the two- to three-fold increase in the frequency of PMGF at Carman appeared to be associated with 275% to 350% higher levels of DTPA-extractable soil Cu levels. We hypothesize that the inability to combine PMGF data across locations in this study may have been due to significantly higher rates of PMGF at Carman possibly resulting from low soil Cu levels. Copper deficiency is known to induce male-sterility in spring wheat by reducing the quantity, size, and starch content of pollen grains (Graham, 1975). The mechanism by which this occurs is thought to be an interruption of microsporogenesis at or near meiosis in Cu-deficient plants (Graham, 1976). A higher frequency of male-sterility induces normally closed florets to open, increasing the opportunity for outcrossing (Virmani and Edwards, 1983; Waines and Hegde, 2003). If reduced levels of soil Cu did promote gene flow at Carman, higher levels of ergot-infected kernels at that location also would be expected as ergot infection requires ascospores to land on and penetrate the ovary to initiate the disease cycle (Bailey et al., 2003). Indeed, significantly more ergot-infected kernels were identified in wheat samples from Carman than Winnipeg (Table 7.4), supporting the hypothesis that increased rates of PMGF at Carman were associated with a reduction in soil Cu concentration. This is the first report of an association between soil Cu level and PMGF in spring wheat and further investigation is required to confirm these findings.

An important objective of this research was to assess the association between flowering synchrony and PMGF to determine if flowering synchrony could be used to predict PMGF *a priori*. The results of this study clearly indicate that flowering synchrony was not a good predictor of PMGF (Table 7.7). Although a weak positive correlation was found at both sites, correlations between flowering synchrony and PMGF were never statistically significant. This may be due to the fact the flowering period in spring wheat is defined as the period during which anthesis or pollen shed occurs (Waines and Hegde, 2003), and thus was quantified as such. While stigmas are most receptive to pollen during

the first 2 to 5 d of anthesis, stigmas have been shown to remain receptive to pollen for up to 13 d after anthesis if self-fertilization fails (De Vries, 1971; Khan et al., 1973). Essentially, visual flowering cues may have ceased long before fertilization actually occurred. Nevertheless, this suggests that flowering synchrony in spring wheat is simply a prerequisite for PMGF, and essentially acts as an "on-off" switch. When flowering synchrony is "off" and no flowering overlap occurs, PMGF cannot occur; however, when flowering synchrony is "on", PMGF can occur, the level of which is dependent on a number of factors including pollen viability, stigma receptivity, male-sterility, distance from the pollen source, environmental and edaphic conditions, and floret characteristics (De Vries, 1971; Waines and Hegde, 2003; Hanson et al., 2005b).

In summary, we have shown that the frequency of PMGF in spring wheat was profoundly influenced by crop density, but showed no dependence on either crop genotype or height. While increasing crop densities beyond the recommended 300 plants m^{-2} provided no obvious benefit to reducing PMGF, our results provide evidence of a critical crop density of 175 to 200 plants m^{-2} below which, the frequency of PMGF rises exponentially with decreasing crop density. Achieving this density will be important for non-transgenic wheat producers for whom limiting the amount genetic admixture in their wheat crops is most prudent. It is important to note that the rates of gene flow we observed were consistently low and certainly well-below the 0.9% threshold required by the European Union for labeling transgenic content. However, given intense selection pressure such as that imposed by a herbicide-resistance trait, the low rates of PMGF observed in this study could have important consequences for coexistence between transgenic and non-transgenic wheat (Brûlé-Babel et al., 2006).

8.0 General Discussion

8.1 Spring wheat response to imazamox

Phenotypic markers are commonly used to identify outcrossing (Poehlman and Sleper, 1995) and have been employed in a number of outcrossing studies involving wheat (Hucl and Matus-Cádiz, 2001; Matus-Cádiz et al., 2004; Hanson et al., 2005a; Gaines et al., 2007a, 2007b; Matus-Cádiz et al., 2007). Results presented in this thesis demonstrate the clear distinguishability of heterozygous hybrids from R and S genotypes in the F_1 generation and indicate that the IR wheat trait is a suitable phenotypic marker for identifying hybrids in gene flow studies (Table 3.3, Fig. 3.2). In fact, given the difficulty of identifying blue aleurone seeds reported by Matus-Cádiz et al. (2007), the IR wheat trait may provide a more discriminatory marker than the blue aleurone marker.

Results from this thesis also demonstrate that spring wheat genotypes respond similarly to imazamox, despite diverse genetic backgrounds (Figure 3.1). This indicates that parental background has little effect on imazamox-resistance in spring wheat and concurs with the findings of Hanson et al. (2006). More importantly, this suggests that future studies utilizing IR wheat as a phenotypic marker should be able to screen for hybrids using a single dose of imazamox, regardless of the combination of genotypes involved. However, the exact dosage required will be a function of the ambient environmental conditions, as well as the number and location of the resistance genes involved (Geier et al., 2004; Frihauf et al., 2005; Hanson et al, 2006).

Imidazolinone herbicides are frequently used in crop rotations due to the development of a number of IR crops including corn, wheat, canola, and sunflower (Tan et al., 2005). In addition, IMI herbicides also can be applied to naturally tolerant grain legumes such as pea (*Pisum sativum* L.) and bean (*Phaseolus vulgaris* L.). Imidazolinone-resistant spring wheat when grown in rotation will outcross to non-IR volunteer wheat, resulting in

transfer of the resistance trait to the volunteer population. Volunteer F_1 hybrid wheat plants exhibiting intermediate levels of imazamox resistance could make control of volunteer wheat more difficult for wheat growers who also grow other IR crops in rotation. Based on the results of our growth room study, current label doses of imazamox for other crops in Canada (which range from 15 g a.i. ha^{-1} to 20 g a.i. ha^{-1}; Anonymous, 2008) are unlikely to satisfactorily control volunteer IR wheat hybrids in the field (predicted 19% to 17% shoot biomass accumulation 21 DAT at these doses). Furthermore, IMI herbicides are often more active indoors as compared to field conditions. For example, Pozniak et al. (2004b) found only slight injury and no significant reduction in yield in IR BW755 at 20 g a.i. ha^{-1} under field conditions, whereas the predicted shoot biomass of CDC Imagine was reduced by 38% in the current study at that dose (Figure 3.1). Moreover, observations have indicated that the majority (86%) of heterozygous imazamox hybrids survived imazamox applications of 20 g a.i. ha^{-1} in the field (personal observation). More research is therefore required to determine the survival and fitness of volunteer IR wheat hybrids when exposed to imazamox under field conditions.

8.2 Temporal isolation of spring wheat crops from volunteer populations

Results presented in this thesis suggest that temporal isolation should be an effective mechanism to minimize volunteer-to-crop gene flow in spring wheat fields. Both flowering synchrony and PMGF peaked when volunteer wheat emergence coincided with crop emergence (Figure 4.3, Fig. 5.3). Moreover, dramatic reductions in both parameters were achieved when emergence and hence, flowering occurred asynchronously. These findings led to acceptance of the main hypothesis that flowering synchrony and PMGF would be highest when volunteer emergence coincided with crop emergence. This concurs with Simard and Légère (2004) who demonstrated that synchrony of flowering between canola and wild radish varied considerably with date of canola sowing. Similar trends also have been observed in corn (Halsey et al., 2005; Della-Porta et al., 2008).

My initial belief, however, was that volunteer density would also be a major determinant of synchrony of flowering and PMGF between volunteer and cropped wheat populations. Although increasing volunteer wheat density did result in a hyperbolic increase in PMGF, these effects were marginal compared with those of emergence timing, and PMGF frequencies were generally below 0.30% (Fig. 4.3). In addition, volunteer density did not significantly affect flowering synchrony in any location or year (Chapter 4). Given that the average density of volunteer wheat across the Prairie provinces is only 6 plant m^{-2} (Leeson et al., 2005), it is appears unlikely that the density of volunteers will be a concern to producers with regards to PMGF and coexistence, particularly if emergence occurs outside of the 125 GDD hybridization window identified in Chapter 5.

It is important to point out that the hybridization window indentified in this research will function to reproductively isolate not only volunteer wheat populations emerging within a 125 GDD interval of the wheat crop, but also adjacent crops emerging within this window as well. Of course the exact magnitude of PMGF will be genotype-dependent, but ensuring that emergence of neighboring crop plants or coexisting volunteer populations does not occur within 125 GDD of crop emergence should minimize PMGF regardless of the genotypes involved. These results are in good agreement with Halsey et al. (2005) who proposed that temporal isolation was an important mechanism to minimize outcrossing in corn, effectively reducing the separation distance necessary to achieve genetic isolation. Likewise, Della-Porta et al. (2008) reported a 50% reduction in transgene flow among corn plots when flowering dates differed by 6 d. In our study, an offset of 3 to 5 d in flowering dates reduced the frequency of PMGF by between 200% and 400% (Table 5.1).

The flowering phenology of individual plants often includes the date of first flowering, the duration of flowering, and the number of flowers produced by the plant at different times of the season (Schmitt et al., 1987). Flowering phenology is often estimated by counting the number of flowers on several representative plants over time to produce a flowering schedule (Elzinga et al., 2007). Synchrony is then estimated by a series of calculations utilizing one of several flowering indices (reviewed in Elzinga et al., 2007).

However, flowering phenology and synchrony studies have often been conducted on natural populations displaying conspicuous flowers. Members of the Graminaceae family are wind-pollinated species lacking conspicuous flowers, therefore necessitating a modification of traditional study methods. The approach to characterizing flowering phenology and synchrony developed herein facilitates high-throughput by allowing a rapid census of several plants in the population. Moreover, the traditional flowering synchrony indices provide a rather crude approximation of flowering synchrony that often requires an arbitrary scale to rate the amount of flowering synchrony (Keatley et al., 2004). Our method provides a deductive, mathematically sound, step-wise approach using a series of calculations to numerically quantify the actual flowering synchrony in a population. To my knowledge, this novel approach has not been previously employed.

8.3 The inability of crop height and density to minimize PMGF where temporal isolation fails

Despite having little effect on flowering synchrony, results presented in this thesis confirm previous reports in natural systems which indicate that PMGF declines with increasing source plant density (Ellstrand et al., 1978; Watkins and Levin, 1990; Morgante et al., 1991). However, the exponential decline that was observed in our study consistently occurred at crop densities below 200 plants m^{-2}, with marginal reductions in PMGF at crop densities above the recommended planting rate of 250 to 300 plants m^{-2} (Manitoba Agriculture, Food, and Rural Initiatives, 2008) (Figure 7.2). These results failed to lend support for the main hypothesis that increasing crop density and growing cultivars that were taller than volunteer plants would minimize both flowering synchrony and PMGF. Because increasing crop density from 300 to 600 plants m^{-2} would double seed costs and neither flowering synchrony nor PMGF were minimized at higher than recommended densities, it can be concluded that currently recommended planting rates optimally minimize PMGF between cropped and volunteer wheat populations. These planting rates are also economically and biologically optimized with regard to seed costs, yield potential and competitive ability (Mason et al., 2007; Otteson at al., 2007). However, it is crucial that plant stands of at least 200 cropped spring wheat plants m^{-2} be

attained as PMGF frequencies rise exponentially with each per plant decrease in crop density below this critical density (Figure 7.2). This will be most important and perhaps most challenging for organic producers who will be forced to minimize transgenic admixture and who may, due to agronomic limitations, experience lower crop densities at flowering more often than in conventional systems. Ultimately, producers in both systems must protect against the low crop densities that accompany crop failure, which unfortunately, often results from the same adverse environmental conditions that also promote outcrossing such as prolonged drought or excessive heat (De Vries, 1971, 1972; Waines and Hegde, 2003).

Results presented in this thesis also suggest that crop height may have merit in reducing flowering synchrony between cropped and volunteer wheat populations (Fig. 6.4). Although this lends support to the initial hypothesis that crop height may influence flowering phenology and synchrony, a firm conclusion cannot be reached due to the limited number of genotypes we included in each height class. Logistical constraints precluded the inclusion of several genotypes in each height class and would have effectively doubled the size of the experiment. Nevertheless, genotypes were chosen randomly within each height class and the consistent grouping (across all site-years) of tall and short genotypes separately with regard to floral characteristics is interesting in itself, but it also begs the question of whether these differences are due to shading or whether they are in fact products of the specific genotypes included in the study? In either case, it is important to note that neither genotype nor plant height had any effect on the frequency of PMGF between volunteer and cropped wheat (Table 7.6, Fig. 7.2). Further research is required, however, using a larger sample of genotypes to elucidate the influence of light quantity and quality on the phenology and synchrony of flowering between wheat populations.

8.4 General considerations and implications for coexistence

Pollen-mediated gene flow estimates reported in this thesis have been derived through careful experimentation using a sub-sampling procedure. To conduct field-scale experiments, sample, screen, and confirm heterozygosity for all established plants would

have been impossible from both a logistical and an economical standpoint. By sub-sampling, we were able to test the hypotheses in accordance with the initial objectives and provide robust estimates of PMGF in the process. However, it is possible that a portion of the unsampled plants were hybrids and thus, we may have under-estimated gene flow in this regard. Further under-estimation may also stem from sowing the volunteer population in rows; however, the benefit of accurately differentiating volunteer plants from those of the crop far outweighs the likely small under-estimation of PMGF that may have resulted. When judging the validity of these results, it is important to recall that an estimate is at best an "approximation of an unknown quantity or outcome based on known information" (Weisstein, 2008). I am confident that the results presented in this thesis represent an excellent approximation of the true frequency of PMGF between volunteer and cropped spring wheat populations and they certainly do not compromise either the responses observed or the conclusions drawn. Moreover, the frequencies of PMGF observed in this thesis are well-within the range reported in the literature for spring wheat (Martin, 1990; Hucl, 1996; Matus-Cádiz et al., 2004; Hanson et al., 2005b). The frequencies of PMGF observed in this thesis although low, should not be regarded as trivial, since selection pressure can rapidly select for an increase in those individuals possessing a fitness advantage in the presence of a selective agent (Brûlé-Babel et al., 2006). Traits conferring evolutionary advantages could include herbicide resistance, drought tolerance, and enhanced nutrient use efficiency. These traits will be difficult to contain where selection pressure is applied or exists naturally.

There is currently little empirical data to relate the degree of flowering synchrony with the level of F_1 hybridization. Theoretically, we would expect that the relative ranking of genotypes by their levels of flowering synchrony with volunteer wheat should match their relative propensity for F_1 hybridization. Therefore, for the genotypes we examined, Amazon and Prodigy should have the highest potential for PMGF. Although not statistically significant (Table 7.6), PMGF frequencies were indeed greatest in Amazon in three of four site-years (data not shown). In contrast, PMGF frequencies in Prodigy were generally lower than those recorded for Vista and Oslo (data not shown). Given that Oslo exhibited negligible flowering synchrony with the volunteer population but higher PMGF

frequencies than other cultivars, this suggests that 1) other variables must be driving outcrossing and 2) a dichotomy may exist between PMGF and flowering synchrony.

Results presented throughout this thesis show that as expected, a similar trend exists between flowering synchrony and PMGF. However, flowering synchrony between volunteer populations continually exhibited a poor correlation with PMGF frequencies, and was not statistically significant in some cases (Tables 5.4 and 7.7). Where significant correlations were detected, flowering synchrony explained only a minor amount of the variation in PMGF data (Figure 5.5). This trend was consistent irrespective of location, year, volunteer emergence timing, crop genotype and height, as well as crop and volunteer density. Taken together, these results provide strong evidence for a dichotomy between flowering synchrony and PMGF. While the cause of this incongruence cannot be pinpointed with certainty given the nature of our studies, a synthesis of the results presented in Chapters 4 and 5 reveals some important insights. Flowering in wheat consists of the presentation and dehiscence of pollen-filled anthers (Waines and Hegde, 2003) and therefore, the quantity of these structures was estimated visually in our studies to facilitate recording data daily on a large number of plots. Because the stigmatic surfaces of wheat florets are known to remain receptive to pollen for up to 13 d if not successfully self-fertilized (De Vries, 1971; Khan et al., 1973), it is conceivable that flowering, as quantified in this study, would be considered complete even though florets may not yet have been fertilized. If this were the case, late-emerging volunteers in Chapter 5 would be expected to pollinate the crop despite very little overlap in their flowering periods. Indeed, PMGF was detected in several plots containing late-emerging volunteers that shared little-to-no flowering synchrony with the crop. Moreover, the model developed in Chapter 5 also predicted little-to-no PMGF in these late emerging volunteers, yet PMGF was identified consistently, regardless of density. Therefore, we can conclude that flowering synchrony can provide an indication of the potential for PMGF, but cannot be used to reliably predict PMGF between spring wheat volunteers and wheat crops. A poor relationship between flowering synchrony and PMGF has also been observed in canola (Marie-Josée Simard, personal communication, 2008).

Results presented in this thesis confirm that gene flow between volunteer and cropped spring wheat can and will occur. Thus, the potential for AP in wheat, despite its primarily self-pollinating nature, is real and perhaps absolute. However, the results reported in this thesis come from small-plot research and despite several attempts to quantify PMGF with increasing distance, no commercial-scale study with a large pollen source exists that would effectively simulate PMGF if transgenic wheat was approved for unconfined commercial release. Therefore, it is not possible at this time to infer with complete certainty regarding coexistence between transgenic and non-transgenic wheat crops. However, this research has identified overwhelming evidence that outcrossing in wheat is a certainty and impurity thresholds of 0% are unrealistic in any commercial-scale context, even with the appropriate temporal and spatial isolation. This is supported by evidence from the field where off-types in western Canadian wheat samples average 0.01 – 0.02% (Hucl et al., 2004), well-below acceptable impurity thresholds for several classes of wheat in Canada but above 0%.

Coexistence, by definition, implies a choice and a guarantee for both producers and consumers but without acceptable and realistic threshold levels for AP, guarantees cannot exist or be met. Critical to the successful introduction and coexistence of transgenic and non-transgenic wheat will be the development of realistic thresholds which can be met and not exceeded on a routine basis. Data presented in this thesis suggest that coexistence between transgenic and non-transgenic wheat may be possible with predefined, science-based, reasonable thresholds developed from sound, accurate mechanistic and stochastic models. The development of such models will permit the exploration of ideas and simulate events such as the potential introduction of transgenic wheat into cropping systems, where it is not possible to field-test for logistical, political, or financial reasons.

These studies also provide compelling evidence that volunteer wheat will serve to facilitate some degree of intraspecific gene movement. Moreover, the densities used in these studies are representative of those observed in western Canadian fields and the results stress the importance of strict volunteer management to ensure threshold levels are met. Taken together, these studies provide an estimate of the potential level of movement of transgenes between cropped and volunteer wheat, vital information for the

development of coexistence models based on population dynamics and gene flow. Because models are inherently limited in their predictive accuracy and precision by the quality of the data and validity of the assumptions on which the model is built, research aimed at providing data for such models must be prioritized (Willenborg and Van Acker, 2006). The findings emerging from this thesis represent a solid contribution to the debate surrounding coexistence between transgenic and non-transgenic wheat. They will also be of use in the development of mechanistic models that predict spatial and temporal gene flow in wheat.

It is likely that transgenic wheat will eventually be introduced into the environment and it is also likely that this introduction will be widespread and unconfined. Increased concern regarding the potential AP of transgenic wheat is inevitable due to the high frequency of wheat in crop rotations, the vast acreage on which wheat is grown, and the large volume of wheat in the grain handling system. Given this reality, the major issues that must be promptly addressed are ensuring cultivar purity by preventing grain commingling, limiting PMGF, and further examination of the potential spread of transgenes including any subsequent ecological implications. Overall, this thesis answered most of the initial objectives, yet research is still required in the area of volunteer wheat seedling ecology, persistence, and population dynamics. Further studies must also be carried out to determine harvest losses both prior to and following mechanical harvesting. More studies are also needed to evaluate several avenues of gene flow between hexaploid wheat and *Ae. cylindrica*, *S. cereale*, *Triticosecale*, and *T. turgidum*, both with respect to the biology and ecology of the two species as well as examining the factors that could contribute to gene flow between them. Studies are needed to determine if gene flow at various levels and distances has a substantial effect on volunteer wheat population dynamics and whether this effect is biologically important. Finally, there remains a critical need for modeling to evaluate a number of aspects in the management of gene flow such as the influence of crop rotation, crop management, and genotype (genetics) on trait movement and confinement in wheat. In this way, we may increase our understanding of the biology and ecology that underlie metapopulation

dynamics in cropped and volunteer wheat with the aim of developing sensible coexistence rules to facilitate the informed introduction of transgenic wheat.

The core of this thesis was based on a research project that was both theoretical in nature and practical in application and in this regard, I believe that the goal has been satisfactorily accomplished. Recommendations can now be made to producers with regard to the critical period (hybridization window) during which the wheat crop must be kept volunteer-free to minimize flowering synchrony and PMGF potential. I am also confident in recommending that producers not increase crop densities beyond the rates currently recommended, but that they target a plant stand of at least 200 spring wheat crop plants m^{-2}. In one respect, the majority of the work in this thesis is theoretical since transgenic wheat has not yet been introduced; however, introduction in the future is likely. On the other hand, the development of a novel analytical method for flowering data was based largely on theoretical mathematics and proved fundamental to estimating the degree of flowering overlap and correlating these results with PMGF. To my knowledge, this has not been employed previously and represents a substantial contribution to the literature on floral ecology.

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Appendix A – Chi-square tests for goodness-of-fit

Table A.1 Segregation of seedlings derived from suspected hybrids for imazamox resistance in the $F_{1:2}$ generation. Data are from Chapter 5.0 examining the influence of volunteer wheat relative time of emergence and density on gene flow between cropped and volunteer wheat populations.

Site	Year	Resistant	Susceptible	Ratio	χ^2	Probability†
		——— no. of plants ———				
Carman	2005	256	72	3:1	1.63	0.202
Winnipeg	2005	188	52	3:1	1.42	0.233
Carman	2006	647	239	3:1	1.95	0.162
Winnipeg	2006	274	110	3:1	2.72	0.099

† Probability values of 0.05 or greater indicate that the data does not differ significantly from the test ratio.

Table A.2 Segregation of seedlings derived from suspected hybrids for imazamox resistance in the $F_{1:2}$ generation. Data are from Chapter 7.0 examining the influence of crop density and height on gene flow between cropped and volunteer wheat populations.

Site	Year	Resistant	Susceptible	Ratio	χ^2	Probability†
		——— no. of plants ———				
Carman	2005	219	61	3:1	1.54	0.214
Winnipeg	2005	136	40	3:1	0.49	0.486
Carman	2006	709	218	3:1	1.13	0.288
Winnipeg	2006	228	86	3:1	1.09	0.296

† Probability values of 0.05 or greater indicate that the data does not differ significantly from the test ratio.