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Globally, the estimated total area planted with transgenic plants producing *Bacillus thuringiensis* (*Bt*) toxins was 12 million hectares in 2001. The risk of target pests becoming resistant to these toxins has led to the implementation of resistance-management strategies. The efficiency and sustainability of these strategies, including the high-dose plus refuge strategy currently recommended for North American maize, depend on the initial frequency of resistance alleles. In this study, we estimated the initial frequencies of alleles conferring resistance to transgenic *Bt* poplars producing Cry3A in a natural population of the poplar pest *Chrysomela tremulae* (Coleoptera: Chrysomelidae). We used the F_2 screen method developed for detecting resistance alleles in natural pest populations. At least three parents of the 270 lines tested were heterozygous for a major *Bt* resistance allele. We estimated mean resistance-allele frequency for the period 1999–2001 at 0.0037 (95% confidence interval = 0.000 45–0.0080) with a detection probability of 90%. These results demonstrate that (i) the F_2 screen method can be used to detect major alleles conferring resistance to *Bt*-producing plants in insects and (ii) the initial frequency of alleles conferring resistance to *Bt* toxin can be close to the highest theoretical values that are expected prior to the use of *Bt* plants if considering fitness costs and typical mutation rates.

Keywords: *Chrysomela tremulae*; resistance-allele frequency; transgenic *Bacillus thuringiensis* poplar; high-dose plus refuge strategy; resistance management; F_2 screen

1. INTRODUCTION

Genetically modified plants containing genes encoding toxins from the bacterium *Bacillus thuringiensis* (*Bt* plants) provide a safe and effective method for pest insect control (Scott & Wilkinson 1998). The estimated global area planted with transgenic plants of all types was 52.6 million hectares in 2001, with 12 million hectares (23%) planted with *Bt* plants (James 2001). The increase in commercialization of these *Bt* plants has magnified the risk of targeted insect pest species rapidly adapting to this ecologically valuable class of toxin (Gould 1998; Wolfenbarger & Phifer 2000). Indeed, *Bt*-resistant strains have been selected under laboratory conditions for several pest species (reviewed by Frutos *et al.* 1999; Sanchis 2000), and field populations of *Plutella xylostella* have already been found to display substantial resistance to *Bt* toxins (Tabashnik *et al.* 1990). Therefore, one of the most important elements in the cultivation of *Bt* plants is the development of effective resistance-management plans, to delay the appearance of resistance to *Bt* toxins in the target pests (Gould 1998). With this aim in mind, governmental agencies, in collaboration with growers' associations and seed companies, have encouraged farmers to implement the high-dose plus refuge strategy (Georghiou & Taylor 1977; Alstad & Andow 1995), predicting that random mating between selected (in transgenic areas) and unselected (in refuges) insect populations can delay the evolution of resistance.

The high-dose plus refuge strategy may be very useful for delaying the evolution of *Bt* resistance. The degree to which this strategy can be expected to delay the evolution of widespread resistance increases as initial *Bt* resistance-allele frequency declines, as is true of other strategies. Evaluation of the initial frequency of resistance has been a challenging task in the last few years. Results from three landmark papers revealed that three lepidopteran pest species—*Heliothis virescens*, *P. xylostella* and *Pectinophora gossypiella*—display high initial frequencies of alleles for resistance to *Bt* crops, calling for a re-examination of the assumptions of resistance-management models (Gould *et al.* 1997; Tabashnik *et al.* 1997, 2000). The experiments described in these papers were, however, subject to a small flaw in that the frequencies of *Bt* resistance alleles were evaluated after the introduction of *Bt* plants and/or in geographical areas previously treated with biopesticide formulations containing *Bt* crystal proteins. Thus, the frequencies reported to date cannot rigorously be taken as actual initial allele frequencies—i.e. the frequencies at which the *Bt* resistance allele segregates in field populations before the introduction of artificial selection pressures resulting from pest management—and as such may

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not adequately reflect the probability of natural pest populations challenging the introduction of *Bt* plants.

Recently, transgenic poplars producing high levels of the *Bt* Cry3A toxin have been produced (Génissel *et al.* 2003). The foliage of these transgenic poplars is highly toxic to *Chrysomela tremulae* (Coleoptera: Chrysomelidae), a polyvoltine oligophagous beetle responsible for massive attacks on native and introduced hybrid poplars (Augustin & Lévieux 1993). *Bt* poplars have not been disseminated and the Cry3A *Bt* toxin produced by these *Bt* poplars has never been used in French agricultural pest-management programmes. This situation provided us with a unique opportunity to evaluate the frequency of *Bt* resistance alleles in natural pest populations prior to the introduction of artificial positive selection pressures. Using the F_2 screen method proposed by Andow & Alstad (1998), we showed that, in a field population of *C. tremulae* surveyed over three consecutive years, the frequency of an allele conferring resistance to *Bt* poplars was 0.0037 (95% confidence interval (CI) = 0.000 45–0.0080).

2. MATERIAL AND METHODS

(a) *F_2 screen*

The F_2 screen method is conducted by: (i) sampling mated adult females from natural populations, and establishing isofemale lines in laboratory conditions; (ii) rearing and sib-mating F_1 progeny in each isofemale line; (iii) rearing eggs from the F_1 parents and screening F_2 neonates for *Bt* susceptibility; (iv) statistical analysis of the data; and (v) retesting of potential positive isofemale lines (Andow & Alstad 1998). As each female carries four haplotypes—two of her own and two from her mate—each isofemale line enables the characterization of four genomes.

(b) *Sampling and sib-mating*

Insects were sampled at a single site ('La Chesnaye', Vatan) located in the Centre region of France. At this site, three groups of adults were collected from young leaves and twigs of hybrid poplars (*Populus deltoides* × *P. trichocarpa* and *P. deltoides* × *P. nigra*) in August 1999, April 2000 and June 2001. To minimize the probability of collecting sib-related adults, adults were homogeneously sampled over the whole surface (*ca.* 1 ha) of the field. Adults sampled in 1999 corresponded to the first generation and those collected in 2000 and 2001 were from overwintering adults (*C. tremulae* being polyvoltine in the Centre region).

The sex of each insect was determined. The males were killed, and each female was isolated and kept in standard laboratory conditions (20 °C under a 16 L : 8 D photoperiod) in 12 cm × 12 cm × 7 cm boxes. The number of F_1 males and females that were sib-mated was recorded. Prior to the F_2 screen procedure, the F_0 and F_1 generations were fed on fresh leaves of a poplar hybrid clone (*P. tremula* × *P. tremuloides*, Institut National de la Recherche Agronomique no. 353-38), grown in the field or in greenhouses.

(c) *Screening procedure*

Egg masses produced during the peak of egg production were collected from F_1 females for a few weeks, and incubated at 15 °C. F_2 neonates emerging from these masses were fed on leaf discs cut from fresh mature leaves of a transgenic *Bt* poplar line placed on moist filter paper to prevent them from drying out. This *Bt* poplar line produces an amount of Cry3A protein equivalent to *ca.* 0.05% of the total soluble protein in mature leaves (Génissel *et al.* 2003). The resulting concentration of Cry3A toxins is lethal to all susceptible *C. tremulae* neonates within 24 h of feeding, as shown by the results of bioassays performed on large samples of individuals from various susceptible laboratory strains (Génissel *et al.* 2003). After 72 h, surviving larvae that had fed actively on *Bt* poplar were classified as resistant larvae.

(d) *Expected proportions of resistant larvae*

If one of the two F_0 parents giving rise to an isofemale line is heterozygous for a *Bt* resistance allele (R), then the expected number of heterozygotes (RS) in the F_1 generation is 50% of the total number of adults. If mating is random, the expected frequency of F_1 heterozygote by heterozygote matings (RS × RS) is 0.25. Within egg masses produced from such matings, the number of resistant homozygotes (RR) is expected to be 25% of the total number of offspring. Therefore, 6.25% of the F_2 larvae should be homozygous and resistant (RR).

If the R allele confers recessive resistance to the amount of Cry3A toxin produced by *Bt* poplars then 6.25% of the F_2 neonates would be expected to survive on *Bt* poplar. From this expected frequency we can make two further predictions: (i) 25% of the egg masses produced by the F_1 females should give rise to resistant larvae (these egg masses are referred to as resistant egg masses); and (ii) the number of resistant larvae should be 25% of the total number of offspring emerging from resistant egg masses.

(e) *Estimating the frequency of the resistance allele and experiment-wise probability*

Expected allele frequencies were calculated using eqn 1 from Andow & Alstad (1998). From their later paper we calculated 95% CIs using eqn 5 if no resistant lines were detected, or eqn 7 if resistant lines were detected (Andow & Alstad 1999). Data for the three samples were pooled by assuming an uninformative beta prior distribution, Beta (u,v), with $u = v = 1$, appropriate when no prior data are available (Andow *et al.* 2000). For each line, detection probabilities were calculated using the algorithm described in Andow & Alstad (1998). This calculation gave the probability of detecting a resistance allele in an isofemale line if the line actually had a resistance allele. This probability is equal to [1 − (probability of a false negative)] and is based on the probability that the resistance allele is lost prior to screening in the F_2 (Andow & Alstad 1998). An experiment-wise probability corresponding to the mean probability of not detecting a resistance allele was calculated for each sample and over the three samples.

3. RESULTS

(a) *Allele frequency in the sample collected in 1999*

Out of the 179 females collected, 128 (72%) produced enough offspring for the production of sib-mated F_1. However, we were able to complete the F_2 screen for only 28 isofemale lines, with a mean ± s.d. of 171.2 ± 94.0 larvae tested per line. The number of F_1 females used to produce the F_2 of each line is indicated in figure 1*a*. Despite the small number of lines tested, one isofemale line (line 126) displayed resistant F_2 larvae.

For this positive line, 2.3% (20 out of 855) of the F_2 neonates tested survived on *Bt* poplar. This frequency was significantly lower (χ^2-test: $\chi^2_1 = 21.65$, $p < 10^{-5}$) than the 6.25% of resistant larvae expected under the assumptions of the F_2 screen. When the proportion of resistant larvae

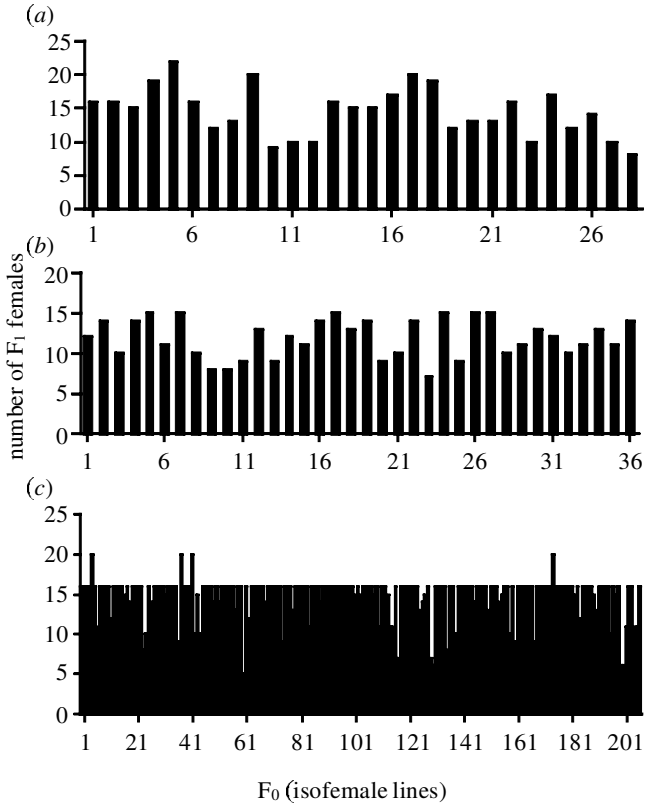

Figure 1. Number of F_1 females per F_0 during F_2 screen experiments in (*a*)1999 (28 F_0); (*b*) 2000 (36 F_0); and (*c*) 2001 (206 F_0). The mean ± s.d. number of F_1 females was 14.46 ± 3.65, 11.83 ± 2.38 and 14.92 ± 2.50 in 1999, 2000 and 2001, respectively.

was corrected by incorporating the mortality level on non-*Bt* poplar (14.4%, $n = 125$), the frequency was 2.7%, which is still significantly lower than the expected frequency (χ^2-test: $\chi^2_1 = 14.86$, $p < 10^{-5}$). This lower frequency resulted from the proportion of resistant larvae per resistant egg mass being lower than expected. Indeed, the proportion of resistant egg masses was 33% (12 out of 36), consistent with the expected proportion of 25% (χ^2-test: $\chi^2_1 = 0.93$, $p = 0.24$). Conversely, the mean frequency (± s.e.) of resistant larvae per resistant egg mass, corrected according to the mortality level on non-*Bt* poplar, was 9.9 ± 1.8%, a value significantly lower (χ^2-test: $\chi^2_1 = 37.61$, $p < 10^{-5}$) than the expected value of 25%. This may be caused by a fitness cost associated with the R allele. Alternatively, *Bt* poplar may produce enough toxin to kill a fraction of the RR individuals. Line 126 was retested over two more generations. Resistant larvae accounted for 2.1% (30 out of 1414) and 0.9% (13 out of 1425) of the F_3 and F_4 larvae tested, respectively. Isofemale line 126 was therefore considered to be resistant.

The most parsimonious explanation for these results is that one of the parents of isofemale line 126 was heterozygous for a *Bt* resistance allele. Based on Bayesian statistics, the expected frequency of this allele in the sampled population was 0.017 (95% CI = 0.0021–0.044; table 1). More than 80% of the lines had a detection probability of more than 95% (figure 2*a*) and the detection probability calculated over the 28 lines was 92.4% (table 1).

(b) *Allele frequency in the sample collected in 2000*

Out of the 51 isofemale lines collected, 39 (76.5%) produced enough fertile adults for the production of F_1 sib-mated lines. Out of the 36 isofemale lines screened in the F_2 generation, we tested a mean ± s.d. of 393.1 ± 137.0 larvae per line on *Bt* poplar. The number of F_1 females used to produce the F_2 of each line is indicated in figure 1*b*. None of these lines produced F_2 resistant larvae. Bayesian statistics gave an estimated expected allele frequency of 0.0066 (95% CI = 0–0.016; table 1). In other words, the probability that the frequency was less than 0.016 was 95%. We calculated the cumulative probability of detecting a resistance allele and found that in more than 70% of the lines the probability of finding a resistance allele was greater than 95% (figure 2*b*). Over all the lines, the detection probability was 86.4% (table 1).

(c) *Allele frequency in the sample collected in 2001*

The last collection of 300 isofemale lines in 2001 generated 252 (84%) isofemale lines from which offspring could be produced by F_1 sib-mating, resulting in 206 isofemale lines that were tested in the F_2 generation (table 1). The number of F_1 females used to produce the F_2 of these lines is given in figure 1*c*. We screened a mean ± s.d. of 514.8 ± 255.2 larvae per line. Three isofemale lines (lines 15, 60 and 116) produced resistant larvae.

All the resistant larvae obtained in line 15 were detected in a single egg mass: nine resistant larvae out of 39 larvae tested. (A total of 90 egg masses (2124 larvae) were tested for line 15.). The survival of these larvae is probably not caused by a decrease in the toxin-expression level in the *Bt* poplar. First, the toxin gene is constitutively expressed in the plant, and preliminary bioassays have shown that it is lethal to susceptible larvae regardless of leaf age. Second, the same transgenic clone (INRA no. 353-38) was always used to feed the larvae. Third, the F_2 screening procedures stimulated a very similar response in all experiments: the susceptible larvae were killed within 24–48 h as were the 30 larvae in the 'resistant' egg mass of line 15. Unfortunately, all the resistant larvae died before the adult stage. Moreover, we detected no further resistant larvae when this line was retested in the F_3 and F_4 generations from unselected F_2 larvae. Line 15 was therefore considered to be a false positive.

In line 60, resistant larvae accounted for 7.9% (171 out of 2157) of all the F_2 larvae tested. If corrected by incorporating the mortality level found on non-*Bt* poplar (11.4%, $n = 44$), the proportion of resistant larvae was 8.9%. This frequency is significantly higher (χ^2-test: $\chi^2_1 = 23.28$, $p < 10^{-5}$) than the 6.25% expected under the assumptions of the F_2 screen. This was because the proportion of resistant egg masses was 42% (36 out of 85), a proportion significantly higher (χ^2-test: $\chi^2_1 = 12.74$, $p = 0.0002$) than the expected proportion of 25%. Nevertheless, the mean ± s.e. frequency of resistant larvae per resistant egg mass, once corrected by the mortality level found on non-*Bt* poplar, was 20.8 ± 1.6%. This value is significantly lower (χ^2-test: $\chi^2_1 = 7.71$, $p = 4.8 \times 10^{-3}$) than the expected value of 25%, as for the 1999 resistant line. We retested line 60 in the next generation and resistant larvae accounted for 2% (35 out of 1710) of all the F_3 larvae tested. We concluded that line 60 was a true resistant line.

Table 1. Estimated expected frequency ($E[p]$) of the allele conferring resistance into *Bt* poplar *C. tremulae*.

	number of lines				estimated R allele frequency		
year	F_0	F_1	F_2	n^a	$E[p]$	95% CI	detection probability (%)
1999	179	128	28	1	0.017	(0.0021–0.044)	92.4
2000	51	39	36	0	0.0066	(0–0.016)	86.4
2001	300	252	206	2	0.0036	(0.000 74–0.0085)	91.2
total	530	419	270	3	0.0037	(0.000 45–0.0080)	90.0

[a] Number of F_2 resistant lines.

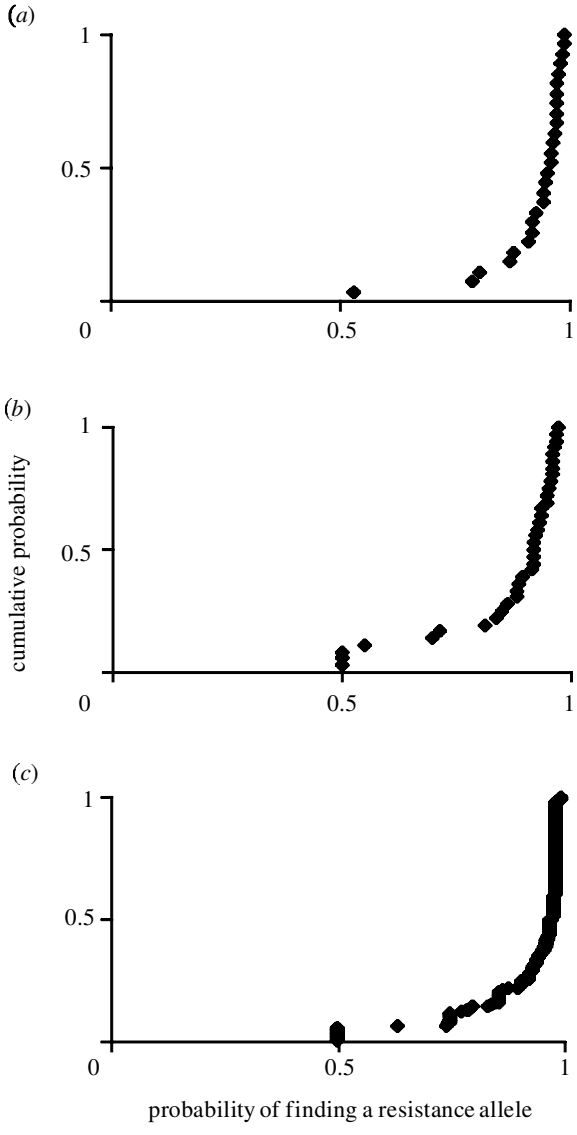

Figure 2. Cumulative probabilities of detecting a resistance allele $\{\Sigma_{i=1}^{N} Ni/N\}$ (N = total number of lines) in isofemale lines tested in (*a*) 1999; (*b*) 2000; and (*c*) 2001.

In the third positive line (line 116) resistant larvae accounted for only 1.9% (64 out of 3411) of all the F_2 larvae tested. When we took the mortality on non-*Bt* poplar (13.3%, $n = 75$) into account, the frequency of resistant larvae was 2.2%. This frequency is significantly lower (χ^2-test: $\chi_1^2 = 83.53$, $p < 10^{-5}$) than the expected value. This was the result of the low proportion of resistant individuals recovered from each resistant egg mass, a tendency also observed for lines 126 (in 1999) and 60. Indeed, in resistant egg masses, the mean ± s.e. frequency of resistant larvae was, once corrected by the mortality level on non-*Bt* poplar, 9.7 ± 1.5%, whereas the expected proportion was 0.25 (χ^2-test: $\chi_1^2 = 98.70$, $p < 10^{-5}$). Conversely, the proportion of resistant egg masses was 21% (24 out of 113), consistent with the expected proportion of 25% (χ^2-test: $\chi_1^2 = 0.66$, $p = 0.41$). The F_3 generation was generated by pooling two resistant F_2 males with eight unselected virgin F_2 females. Almost 9% (206 out of 2309) of the F_3 neonates tested survived on *Bt* poplar. Based on these results, isofemale line 116 was also considered to be resistant.

We therefore concluded that one parent for each of the two lines 60 and 116 was heterozygous for a major *Bt* resistance allele. Based on Bayesian statistics, the expected frequency of this allele in the sampling population was 0.0036 (95% CI = 0.000 74–0.0085; table 1). The detection probability was greater than 95% for more than 80% of the lines (figure 2*c*) and the detection probability calculated over all the lines was 91.2%.

(d) *Global estimate*

Results from the three samples were pooled to estimate the frequency of the *Bt* resistance allele that segregates in the population sampled in the Centre region of France. This estimation is based on the following assumptions: (i) all the resistant lines had the same *Bt* resistance allele; (ii) within each isofemale line, there was no divided paternity resulting from multiple matings; and (iii) the three samples were collected from the same site, as part of a single panmictic population (Génissel *et al.* 2000). As three resistant lines were detected in a total of 270 lines studied, the frequency of the allele conferring resistance to *Bt* poplar was 0.0037 (95% CI = 0.000 45–0.0080; table 1). The detection probability associated with this estimate is 90% (table 1).

(e) *Life cycle of resistant larvae on* Bt *poplar*

One of the three resistant lines (line 60) was further studied to determine whether or not resistant larvae were able to grow to adults when fed on *Bt* poplar only. Sixty resistant larvae were fed exclusively on *Bt* poplar. Twenty individuals (33%, 16 females and four males) reached the adult stage. These adults were pooled and gave birth to more than 25 fertile egg masses. The first three egg masses that were tested by the *Bt* poplar feeding test as for the F_2 screen procedure contained resistant larvae. We therefore concluded that the *Bt* resistance allele detected in line 60 confers a sufficient decrease in susceptibility to the Cry3A

Bt toxin that resistant beetles can complete their entire life cycle on *Bt* poplar.

4. DISCUSSION

Our results indicate that alleles enabling *C. tremulae* larvae to survive and reproduce on *Bt* poplar were segregating in a French poplar stand. At least three parents of the 270 lines tested were heterozygous for a major *Bt* resistance allele. The mean resistance-allele frequency for the period 1999–2001 was 0.0037 (95% CI = 0.000 45–0.0080), with a detection probability of 90%. This frequency is close to the highest values that are expected prior to the introduction of pesticide in the field, when considering fitness costs and typical mutation rates (Roush & McKenzie 1987).

High frequencies of alleles conferring resistance to *Bt* plants have already been detected in field populations of *P. xylostella* (Tabashnik *et al.* 1997), *P. gossypiella* (Tabashnik *et al.* 2000) and *H. virescens* (Gould *et al.* 1997). However, these frequencies may have been artificially increased by man-made changes to the environment.

The R allele frequency of 0.12 reported for a susceptible strain of *P. xylostella* by Tabashnik *et al.* (1997) was obtained in a sampling carried out on Hawaii, where cabbage, broccoli and watercress fields have been treated with *Bt* for many years (Tabashnik *et al.* 1990). Furthermore, even with stringent measures, Tabashnik *et al.* (1997) recognized that a few individuals from *Bt* resistant strains may have occasionally contaminated this susceptible strain.

Cry1Ac-resistant larvae of *P. gossypiella* were recovered in populations captured in Arizona between 1997 and 1999 (Tabashnik *et al.* 2000). However, in 1996, genetically modified cotton producing the Cry1Ac toxin was planted on 730 000 ha of US farm land (Tabashnik *et al.* 1997), and *Bt* cotton accounted for more than half of the more than 100 000 ha of cotton in Arizona in 1997, 1998 and 1999 (Tabashnik *et al.* 2000).

In *H. virescens* the frequency of the allele conferring resistance to *Bt* cotton was calculated for field-collected males captured in 1993 (Gould *et al.* 1997). This frequency may therefore correspond to genuine initial conditions prior to the first commercial planting of transgenic cotton. However, *Bt* sprays were used in cotton fields in the mid-south of the US for a short period of time, and most of the samples screened came from this region (F. Gould, personal communication). Although local entomologists felt that these sprays were ineffective, they may have had sub-lethal effects on *H. virescens* populations, encouraging the development of resistance.

To date, *Bt* poplars have been planted only in a strictly protected insect-proof 20 m² greenhouse located *ca.* 100 km away from the sampling site, and French poplar plantations have never been treated with *Bt* sprays. *Chrysomela tremulae* feeds only on poplars (Augustin & Lévieux 1993) and could not have been exposed to *Bt* toxins from the treatment of other crops because formulations containing the Cry3A toxin—or any related toxin active against Chrysomelidae—have not yet been put on the market in France. Moreover, the migration of artificially selected *Bt* resistant genotypes originating in any bordering country is unlikely as there are neither *Bt* treated poplars nor *Bt* transgenic poplars within the poplar fields of these countries. Thus, our results provide the best evidence yet that alleles conferring resistance to *Bt* plants may be present at detectable frequencies in pest populations prior to any artificial selection resulting from pest management by humans.

Alleles conferring pesticide resistance may be part of the existing genetic variation prior to pesticide treatment, and may be generated by means of recurrent mutations (ffrench-Constant 1994; Andreev *et al.* 1999) and/or migration events (Raymond *et al.* 1991; Guillemaud *et al.* 1996). As individuals carrying these alleles often pay a fitness cost in the absence of pesticide (Roush & McKenzie 1987; Coustau *et al.* 2000), the alleles are expected to segregate at a mutation–selection balance prior to selection. This frequency is *ca.* u/hs, with u being the mutation rate, s the fitness cost and h the dominance of this cost (Hartl & Clark 1997). In such conditions, how is it possible for a *Bt* resistance allele to be present at a frequency of greater than 10^{-3}? One possibility is a combination of a high mutation rate (e.g. $u = 10^{-5}$) with a low (e.g. $s = 0.01$) and/or recessive (e.g. $h = 0.1$) fitness cost. It is also possible that resistance to *Bt* is naturally selected in field populations of *C. tremulae*. The ecological characteristics of *Bt* are largely unknown but this bacterium has been shown to be pathogenic to insects: although rare, natural epizootics do occur in field conditions (Damgaard 2000). This bacterium is present not only in dead insects, but also in soils (e.g. Chilcott & Wigley 1993; Chaufaux *et al.* 1997), water (e.g. Iriarte *et al.* 2000) and the phylloplane of many plants (e.g. Smith & Couche 1991; Mizuki *et al.* 1999). *Bt* strains producing the Cry3A toxin have not yet been shown to be present in poplar plantations or in dead insects of *C. tremulae*, but if such strains were shown to be present, they provide a natural source of selection for the *Bt* resistance allele recovered in this study.

As alleles conferring resistance to insecticidal proteins are not necessarily rare, it is essential to evaluate their frequencies before deploying transgenic plants producing these insecticidal proteins. The discriminative-dose assay approach (consisting of a selection at high *Bt* dose able to discriminate resistant phenotypes (e.g. Roush & Miller 1986; Sims *et al.* 1996)) is not sensitive enough because resistance to *Bt* toxins is often recessive (Bourguet *et al.* 2000; Ferré & Van Rie 2002). This is apparently the case in *C. tremulae*: the proportion of resistant larvae recovered in the three resistant lines suggests that resistance to the doses of Cry3A produced by *Bt* poplar is recessive (preliminary results of crosses between the resistant three lines and a susceptible line also indicate that resistance to *Bt* poplar is recessive). With recessive resistance and genotypic frequencies at Hardy–Weinberg equilibrium, the frequency of resistant individuals in a population equals the square of the allele frequency. Based on our estimates, we would therefore need to assay *ca.* 100 000 *C. tremulae* larvae to find one resistant individual; this number is probably greater than the number of individuals in the population in the poplar stand that has been considered here. In fact, the discriminative-dose assay approach is relevant only for detecting or monitoring alleles that are already at a very high frequency, as in natural populations of *P. gossypiella,* in which the frequency of the recessive Cry1Ac resistance allele may be as high as

0.16 (Tabashnik *et al.* 2000), or that confer dominant resistance to *Bt* toxins.

A more sensitive technique for estimating the initial frequency of *Bt* resistance was developed by Gould *et al.* (1997). Field-collected males of *H. virescens* were mated with virgin homozygous resistant females from a resistant laboratory strain. The genotypes of the offspring were determined by discriminating-dose assay, so that the number of male parents that carried the resistance allele could be inferred. However, this single-pair mating design is effective only if a resistance allele has already been identified and fixed in a resistant strain, a condition that may not be easily satisfied when considering the initial situation before intervention. Moreover, this technique cannot take multiple resistance genes into account (Andow & Alstad 1998).

In this study, we used the F_2 screen developed by Andow & Alstad (1998) and refined by Andow & Alstad (1999). This screening procedure increases the likelihood of detecting recessive and rare resistance alleles over the other two screening procedures cited. In particular, the F_2 screen can be used to estimate the frequency of any resistance allele sampled from the natural population and is suitable for estimating the statistical robustness of any experiment (see Andow & Alstad (1998) and Venette *et al.* (2000, 2002) for a more detailed comparison of the various screening methods). The feasibility of this method has been demonstrated and it has been used to estimate the frequencies of *Bt* resistance alleles in field populations of the European corn borer, *Ostrinia nubilalis* (Andow *et al.* 1998, 2000; Bourguet *et al.* 2003), and the rice stem borer, *Scirpophaga incertulas* (Bentur *et al.* 2000). Although partial *Bt* resistance alleles, conferring a level of resistance not sufficiently high for survival on *Bt* plants producing large amounts of toxin, have been identified in populations of these pest species, major *Bt* resistance alleles have not been detected. These 'negative' results and those reported by Zhao *et al.* (2002) shed some doubts on the ability of the F_2 screen to detect low frequencies of R alleles. The US Environmental Protection Agency therefore waited for data to emerge before using this screening procedure in pest-management programmes (Environmental Protection Agency 2001). Our study demonstrates the usefulness of the F_2 screen for recovering major resistance alleles from pest populations, and validates this technique for monitoring the evolution of recessive *Bt* resistance alleles.

Genetic models have indicated that an allele conferring a recessive resistance at a frequency similar to that reported here for *C. tremulae* could lead to the rapid evolution of resistant populations in the absence of refuges for susceptible individuals (e.g. Mallet & Porter 1992; Alstad & Andow 1995). Conversely, if refuges are planted over 50% of the cultivated area, the evolution of *Bt* resistance can be delayed by up to 20 generations (Alstad & Andow 1995). If the resistance is completely recessive, a refuge as low as 5% might still delay the evolution of resistance for at least 50 generations (Roush 1997). Moreover, our results suggest that resistant beetles may not be as fit as susceptible beetles. During the F_2 screen, the proportion of resistant individuals was lower than expected and only 33% of the resistant beetles completed their life cycle when feeding exclusively on *Bt* poplar. These data suggest that the resistance allele induced a fitness cost and/or conferred an incomplete resistance to *Bt* poplar. Carrière & Tabashnik (2001) have shown that these two factors may prevent and even reverse the evolution of resistance, even if the initial frequency of *Bt* resistance is 0.1. Therefore, rather than challenging the high-dose plus refuge strategy, the results found in the present study provide empirical support for theoretical models using 0.001 and values slightly larger or smaller for the initial resistance-allele frequency.

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