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North American Plant Protection Organization
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NAPPO Science and Technology Documents

ST 03: Application of biological control of the emerald ash borer (EAB) in North America

**Prepared by the members of
NAPPO Technical Advisory Group on EAB**

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October, 2013

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Index

	Page
1. General Biology and Management	3
2. Biological Control Options for Emerald Ash Borer	6
2.1 Classical biological control.....	6
2.1.1 Species of Hymenoptera approved for release in the United States and current program status	6
2.1.2 Biological information	7
2.1.3 US release data.....	8
2.1.4 US recovery data.....	12
2.1.5 Mass-rearing facility – Methods development and process improvements.....	13
2.2 Native parasitoids and predators of EAB	14
2.3 Biological control using entomopathogenic microorganisms.....	16
2.3.1 Biological control with entomopathogenic fungi	17
2.3.2 Mode of action of entomopathogenic fungi	17
2.3.3 Most significant results of entomopathogen research for EAB	17
3. Regulatory processes associated with the importation, the rearing, the release and the domestic movement of biological control agents of insect pests	21
3.1 Regulatory processes in the United States	21
3.2 Regulatory processes in Canada.....	23
3.3 Regulatory processes in Mexico	25
4. Present state, use, and perspectives of the genus <i>Fraxinus</i> in Mexico	26
5. How to Stimulate Research for the Application of Biological Control.....	27
6. Management of EAB through Harmonization of Biological Control Efforts	28
6.1 Exchange of biological control experience.....	28
6.2 Operational adjustments	28
6.3 Centralized repository of strategic knowledge	29
7. Conclusions and Recommendations	29
8. References	29

1. General Biology and Management

The emerald ash borer (EAB) (*Agrilus planipennis* Fairmaire) (Coleoptera: Buprestidae) is an wood-boring insect that has invaded and become established in North America (Haack et al. 2002). EAB is native to large areas of northeastern Asia including the Russian Far East, Korea, China, Japan and Mongolia (Bray et al. 2011). However, in those countries, EAB is not reported as a pest of economic importance (Cappaert et al. 2005). In a field survey in some areas of its native range, the EAB was rare and difficult to find (Schaefer 2004). The EAB was probably transported to North America in solid-wood packing material (Cappaert et al. 2005).

The EAB was discovered in 2002 in Detroit, Michigan and in Windsor, Ontario (Haack et al. 2002). How and when this insect was introduced into North America is unknown but evidence suggests that EAB was present in Michigan for at least 10 years prior to its discovery (Cappaert et al. 2005). This insect is now found in 18 USA states and two Canadian provinces (www.emeraldashborer.info). The states regulated in the USA are: Connecticut, Illinois, Indiana, Iowa, Kansas, Kentucky, Maryland, Massachusetts, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Tennessee, Virginia, Wisconsin and West Virginia. In Canada, this insect is found in Ontario and Quebec. Millions of ash trees in urban, rural and forested settings have been killed and the ecological and economic impacts are increasing as the insect spreads (Poland 2007, Kovacs et al., 2010). Taking into account that the insect is already established in a large area of North America, eradication is no longer practical (Johny et al. 2012a; Poland and McCullough 2010).

In North America, all 20 native species of ash trees (Wallander 2008) are presumably highly vulnerable to EAB, although blue ash (*Fraxinus quadrangulata*) is less susceptible (Tanis and McCullough 2012). Green ash (*F. pennsylvanica*) is typically attacked first in mixed stands and is generally more susceptible than white ash (*F. americana*) trees (Cappaert et al. 2005), although both ultimately succumb to EAB. This insect is considered the worst tree-killing insect introduced into North America (McCullough and Mercader 2012). In northeastern China, the EAB is recorded as attacking preferentially the introduced North American ash species (*F. pennsylvanica* and *F. velutina*) and rarely attacking Asian species such as *F. mandshurica* and *F. chinensis* (Duan et al. 2012b) unless out of their natural forest setting (Liu et al. 2003).

Trees of different sizes can be subject to attack by EAB. Generally, we consider that the attack initially starts in the top of the canopy of large trees followed later by colonization of the main trunk after tree decline (Cappaert 2005). When larvae are present at high densities under the bark, the tree is killed by larvae consuming phloem and outer xylem, and thus girdling the tree, disrupting water and photosynthate transport, starving the roots of nutrient resources and dehydrating the crown (Dean et al. 2012). Death of the trees is observed after 1-3 years of damage (Poland and McCullough 2006; Cappaert et al. 2005).

Apparent symptoms of an EAB infestation on a tree are crown dieback, bark deformities, woodpecker attacks, and epicormic shoot production, and these symptoms become more visible on the tree as the infestation progresses (Cappaert et al. 2005). Typical "D"-shaped exit holes and serpentine galleries under the bark are generally indicative of attack by EAB.

In the Great Lakes area, EAB adults emerge from late May to late July. After emergence, adults feed on foliage over 5-7 days before mating and then females feed another 5-7 days before ovipositing (Cappaert et al. 2005). EAB feed during their entire adult life span (Bauer et al. 2004a) and females can perform multiple matings (Lyons et al. 2004). Each female lays an average of 71 eggs (Wei et al. 2007) on the bark or under bark scales of branches or trunk of the host tree. However, one female held in captivity was able to lay 258 eggs during a 6-week period (Lyons et al. 2004). Under current rearing methods, individual EAB typically produce between 200-300 eggs, with a minority of individuals laying 600-700 eggs over a 10-12 week adult life span (Lelito, unpublished data). After 10-14 days (depending on weather conditions), eggs hatch and larvae

bore toward the phloem and feed and develop through four instars (Cappaert et al. 2005) before reaching the prepupal stage. Larvae feed for many months on the phloem and outer sapwood, and eventually girdle and kill the trees if galleries are extensive (Duan et al. 2010). If larval development has not reached the prepupal stage in fall, then another winter will be required to reach the adult stage. A proportion of larvae will have to overwinter for a second time before their final metamorphosis to the adult stage. Larval development takes longer on healthy and newly attacked trees than on unhealthy trees (Cappaert et al. 2005). During the winter months, earlier instars are generally more prevalent on lightly infested trees whereas EAB larvae in heavily infested trees are largely in the prepupal stage at this time (Cappaert et al. 2005).

The EAB can be spread in two ways: via transportation of infested ash wood and adult flight. Early observations in Michigan suggested that dispersal in low-density outlier sites was less than 1 km/yr (Cappaert et al. 2005). However, in laboratory studies, 20% of EAB mated females flew over 10 km in 24 h on computer-monitored flight mills and 1% flew more than 20 km in 24 h (Taylor et al. 2010).

Different biotic factors have been observed to reduce EAB survival in its native range as well as in North America. In a survey of natural enemies done by Liu and Bauer (2006) in Michigan, it was observed that about 2% of larvae were infected by an entomopathogenic fungus. Commercial and native isolates were tested against EAB larvae and adults, and will be discussed in Chapter 4.

EAB is attacked by a complex of parasitoids in its native range, and field surveys in Michigan, Pennsylvania, Ohio and Ontario have shown that native Hymenoptera also attack the larvae of EAB (Bauer et al. 2004b; Lyons 2010; Cappaert and McCullough 2009; Duan et al. 2009; Kula et al. 2010; see Chapter 3).

Three species of parasitoids that attack the EAB in China were evaluated for their inclusion in a classical biological control program and approved for release in the USA. Two are larval parasitoids, *Spathius agrili* Yang (Hymenoptera: Braconidae) and *Tetrastichus planipennis* Yang (Hymenoptera: Eulophidae), and one is an egg parasitoid, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Liu et al. 2003; Dean et al. 2012). More details are provided in Chapter 2. The results of these studies support the idea that suppression by natural enemies is the most suitable long-term pest management strategy for introduced pests (Hajek et al. 2007; Johny et al. 2012a) such as EAB.

Field studies in recently invaded areas of the USA have shown that woodpeckers are important predators, consuming between 32 and 90% of late instar EAB, prepupae and pupae (Cappaert et al. 2005; Lindell et al. 2008, Duan et al. 2012a). In the native range, woodpeckers do not seem to be an important biotic factor regulating EAB populations. In a survey done in the Russian Far East, predation by woodpeckers on EAB individuals was 3% on Oriental ash (*F. mandschurica*) and 0-41% on North American green ash (*F. pennsylvanica*) (Duan et al. 2012b).

Host tree resistance has been shown to influence survival of EAB (Rebek et al. 2008; Duan et al. 2010; Duan et al. 2012b). In the Russian Far East region, Duan et al. (2012b) observed in a field survey that the EAB was mostly associated with the introduced North American green ash (*F. pennsylvanica*) tree. Moreover, EAB densities were several-fold higher on green ash than on artificially stressed Manchurian ash or Oriental ash. These authors observed EAB cadavers covered with plant callus tissues and considered this plant response as an expression of tree resistance that was higher on Manchurian ash than introduced North American ash. Duan et al. (2012b) thus concluded that host tree resistance might play a major role in suppressing EAB in its place of origin. (However, although initially considered as a physiological trait of Asian species, this resistance was also observed on North American trees: in natural ash forest stands in Michigan, host tree defence was the major source of mortality for EAB larvae (Duan et al. 2010).

Mortality due to low winter temperatures may limit EAB population spread. Studies performed on EAB from southern Ontario demonstrated that prepupae do not survive below an average temperature of -30°C and are considered freeze-intolerant (Crosthwaite et al. 2011). Moreover, the lowest freezing temperature (supercooling point) measured in Ontario prepupae was -35.3°C , lower than the -29.6°C reported from China (Crosthwaite et al. 2011). Studies are required to determine if early instars have the same cold resistance.

Management of the EAB in North America is based on different strategies, namely detection, population reduction attempts and legislative regulation. Federal organizations (USDA-APHIS and CFIA) perform inspection and scouting in high-risk areas. Infested or suspect trees are identified according to typical signs and symptoms, which include bark splits, emergence holes, serpentine-shaped larval feeding galleries, canopy appearance, and adventitious branching. In addition to these visual surveys to delineate EAB infested areas, insect traps are used to detect EAB adults (Crook et al. 2008). In Canada, adhesive-coated green prism traps are installed in the tree canopy and baited with a kairomone, (Z)-3-hexenol. In the USA, similar surveys are underway using purple prism traps baited with manuka oil and (Z)-3-hexenol (USDA-APHIS 2012). Recently an EAB-produced pheromone was synthesized and tested (Silk et al. 2011). New methods under development use the pheromone to synergize the kairomone lure to enhance trap captures (Ryall et al. 2012).

When infested trees are discovered, the cryptic habits of larvae limit control to the following strategies: mechanical control by tree removal and disposal, or systemic injection of insecticides if the tree is not too severely infested. Trees can be cut down and processed to kill the next emerging generation. Chipping the wood or processing it into lumber are ways to recycle trees into valuable products (Wisconsin 2009).

Some insecticides are registered for use against EAB to protect uninfested or lightly infested trees. They can be classified into four categories: systemic insecticides applied as soil injection or drenches; systemic insecticides applied as trunk injections; systemic insecticides applied to the lower trunk; and protective cover sprays applied to the trunk, branches and foliage. Systemic insecticides are translocated between trunk and branches by the vascular system, thus reducing environmental hazards (Hahn et al. 2011). In the USA, different active ingredients are available in different formulations. Depending on the product, some of the active ingredients are imidacloprid, dinotefuran or emamectin benzoate (Herms et al. 2009). However, not all products are equally effective against the EAB (Herms et al. 2009). In Canada, only three products are registered and all are systemic insecticides: TreeAzin® (azadirachtin), Acecap 97® (Acephate - O,S-dimethyl acetylphosphoramidothioate) and Confidor® (Imidacloprid).

In reference to biopesticides, some isolates, which have been demonstrated to be highly virulent against the EAB, can be applied on tree trunks as a pre-emergence trunk sprays (Liu and Bauer 2008a, b) or post-emergence sprays to bark or foliage (Castrillo et al. 2010a). Commercial formulations of fungal entomopathogens will be described in Chapter 4.

From the point of view of regulatory control, the objectives of federal regulatory agencies in the USA (USDA-APHIS) and Canada (CFIA) are to find and delineate new infested areas, legislatively prohibit the movement of specific materials including any ash wood material and firewood of all species from infested to non-infested areas, and organize information campaigns to slow the spread of this invasive insect.

Using all the tools available to deal with the EAB, the move is towards long-term integrated management of EAB populations. An interesting and integrated approach is the pilot study named Slow Ash Mortality (SLAM) that is underway in Michigan (Poland and McCullough 2010). The objective of SLAM is to reduce EAB populations and consequently slow the progression of its

spread. To slow the spread, there must be strong regulatory efforts, cooperation from residents in affected areas, improved detection and suppression methods, and better knowledge of population dynamics and host resistance. Improving the efficacy of each strategy could make a difference in the battle against the EAB.

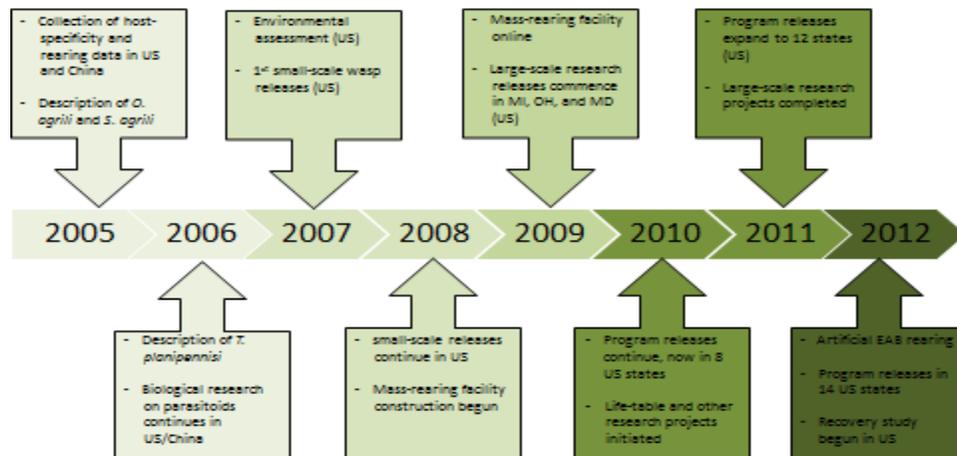
2. Biological Control Options for Emerald Ash Borer

The following sections outline classical biological control (2.1), native parasitoids and predators of EAB (2.2), and entomopathogens of EAB (2.3).

2.1 Classical biological control

Figure 2.1 provides a visual timeline of US efforts to identify, assess, and rear classical biological control agents for EAB. This process, begun in 2005, has begun to bear fruit, as increasing numbers of the agents are being recovered. Sections 2.1.2-2.1.6 provide more detail on the agents themselves, their biology and behavior, numbers of insects released, US release and recovery locations, and a summary of the anticipated future direction of the US biological control program for EAB.

Figure 2.1. Historical timeline of US EAB-biological control program



2.1.1 Species of Hymenoptera approved for release in the United States and current program status

Upon initial detection of EAB in 2002, it was determined that APHIS would pursue exploration for biological control agents and this work was undertaken in conjunction with USDA-Forest Service (Federal Register 2007). Three species of parasitoids, new to science, were collected from China and studied in the laboratory, with the primary focus of early work on behavior, host-specificity, and phenology (Yang et al. 2005, 2006; Zhang et al. 2005).

Subsequent to finding the new species in China, samples were sent to the United States and the process of host-specificity testing was begun at laboratories operated by the U.S. Forest Service and the Plant Protection and Quarantine's Center for Plant Health Science and Technology. An Environmental Assessment project was initiated to determine key aspects of concern regarding the importation and release of the parasitoids, including: climate matching, host-specificity studies to determine if the parasitoids would potentially be a threat to native species if released, and the likely outcome of the default "no action" (e.g. no biological control agents pursued) strategy (USDA-APHIS/FS 2007). Although both *O. agrili* and *S. agrili* were found to attack a small number of

native North American species in the genus *Agrilus*, the rate of attack was low and the preference was for EAB wherever two hosts were offered simultaneously. The overall conclusion of the report was a Finding of No Significant Impact, and the parasitoids were subsequently approved for release in the US (USDA–APHIS/FS 2007).

Most recently, a new species of *Spathius* (*Spathius galinae*; Belokobylskij et al. 2012) has been described and collected from Russia. *S. galinae* is currently in the environmental assessment phase, undergoing host-specificity testing. The hope is that it will meet host-specificity requirements, as it is suspected (based on its more northern range) to have a higher cold-tolerance than *S. agrili*, and thus might be better suited to the northern US and Canada, where ash, especially *F. nigra*, is abundant.

As an overview, the current status of the US classical biological control program is as follows: *Spathius agrili*, *Tetrastichus planipennisi*, and *Oobius agrili* are approved for release and have been released and recovered throughout the EAB infestation in the United States (see 2.1.2, 2.1.3); *S. galinae* is proposed for release and undergoing host-specificity testing; recovery and establishment programs for the three approved parasitoids are underway in many states in the US (see 2.1.4), and the EAB Biological Control Facility is online and successfully producing parasitoids for distribution to federal and state agencies for release into the field (and, to a lesser extent, to support program-relevant research).

2.1.2 Biological information

The three species of parasitoids approved for release into the US are *Oobius agrili* Zhang and Huang (a solitary parthenogenetic endoparasitoid in the family Encyrtidae), *Spathius agrili* Yang (a gregarious idiobiont ectoparasitoid in the family Braconidae) and *Tetrastichus planipennisi* Yang (a gregarious koinobiont endoparasitoid in the family Eulophidae).

The three species of parasitoids all share EAB as their primary host. However, the life stages attacked and the timing of said attack vary between the three species. Two species, *O. agrili* and *S. agrili*, have an obligatory diapause (Liu et al. 2007; Belill and Lelito 2011) and appear to be closely synchronized with EAB emergence. In contrast, *T. planipennisi* does not exhibit diapause behavior (Coggins and Lelito 2011; Duan et al. 2011a), and attacks EAB continuously throughout the warm months of the year. Because of these differences, different biological and ecological aspects are taken into account for each species to determine the timing of releases of each parasitoid in each geographic area. Furthermore, the process of distribution of insect material across the infested area requires constant monitoring of local weather conditions and the accumulation of growing degree-days.

Oobius agrili attacks EAB only at the egg stage, and likely completes two generations a year across most of the range of EAB in the US (Liu et al. 2007). From a rearing perspective, *O. agrili* can be stored, in diapause, for up to 12 months without incurring significant mortality. Adults emerge synchronously within 2-3 of one another weeks following warm-up in the laboratory and begin to reproduce immediately. Thus, this species is perhaps the “easiest” to mass rear for release given a constant supply of EAB eggs as host material (see 2.1.5). Furthermore, the Rearing Facility has developed an extremely efficient method of distributing the parasitoids as pupae within hosts on filter papers, rather than as live adults, which are extremely small and thus highly fragile during shipping (see 2.1.5).

Spathius and *Tetrastichus* attack EAB larvae, with both preferring actively feeding fourth (final) instar but being able to accept third instar hosts as well as pre-pupae as well (Liu et al. 2007; Wang et al. 2008; Ulyshen et al. 2010; Duan et al. 2011a). These various studies have examined the preference of the parasitoids for different stages of EAB under natural (field) and artificial (laboratory) conditions, while a recent studies determined that for *S. agrili*, olfactory cues guide the parasitoid to the host tree, after which time acoustic cues from feeding-induced vibrations assist in

host location (Wang et al. 2010). *S. agrili* appears to have moderate to high host-specificity for EAB, and while it is capable of developing on other species of *Agrilus* native to the US, it has a strong preference for EAB hosts (Yang et al. 2008). Furthermore, *S. agrili* is only attracted to volatile compounds from ash, and to a lesser extent willow (Yang et al. 2008); coupled with the results of Wang and colleagues (2010) noted above on attraction to ash as an important first step in host location, this suggests encounter rates with non-target hosts will be very low.

Competitive exclusion has the potential to occur between the two species of larval parasitoids, and there has been debate over the best strategy to pursue when releasing multiple biological control agents for quite some time (e.g. Turnbull and Chant 1961). In the case of EAB parasitoids, one study indicates *Spathius* to be the better competitor (Ulyshen et al. 2010), but differences in host-finding ability caused by the increased vagility of one or the other of a competing pair have been shown to allow two species of parasitoids to coexist while sharing a host resource (“counter-balanced competition”; Zwölfer 1971; Schröder 1974). Additionally, laboratory studies have shown that *T. planipennisi* can differentiate between a previously parasitized EAB from an unparasitized host, and thus may be able to avoid some competition in this way (Yang et al. 2012).

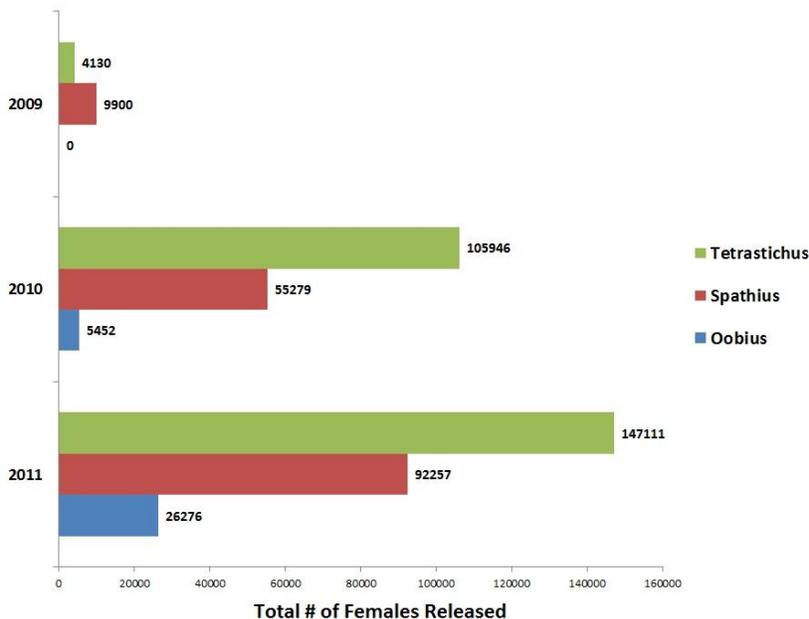
Field studies are recommended to confirm the viability of the US strategy, which is to release both larval parasitoids concurrently at the same site. Field studies thus far have focused on life table data and recovery of the parasitoids following releases (Duan et al. 2012a). At this time, because of limited data on recovery or on any potential regional variation in the success of the larval parasitoids based on climatic factors, the US EAB Program has determined that it is logical to release both larval parasitoids concurrently until sufficient evidence suggests that this decreases success of control of EAB.

2.1.3 US release data

Releases of the three approved parasitoids commenced in small-scale plots in 2007 and continued in 2008. In 2009, the EAB Mass-Rearing Facility came online in Brighton, Michigan, and the process of distribution to infestations in several states began. Most recently, several thousand female *Tetrastichus* have been provided for release in Ontario, Canada, by CFIA to support a pilot biological control project there.

In 2010-2012, the network of release sites in the US expanded considerably. Figure 2.2 shows the progression of total number of wasps released by the Rearing Facility (over 76,000 *Oobius* have been released in 2012; complete data for the two larval parasitoids are not yet available); Tables 2.1-3 provide a breakdown of these releases by US state and county in each year from 2009-2011. Also during this time, the discovery of new EAB infestations, some of which were at least a few years younger than the average age of the EAB infestations at the original parasitoid release sites, provided the opportunity to release parasitoids while EAB population densities were, at least in theory, somewhat less overwhelming when compared to the number of biological control agents released into the environment.

Figure 2.1.: Yearly Totals, 2009-2011, of Female Parasitoids Released in the US



The general release strategy pursued thus far has been to release small quantities (200 females) of each species every other week for five weeks, during two periods each year (1200 females of each species), dependent on the local climate (USDA–APHIS/ARS/FS 2012). Due to variation in parasitoid production at the Rearing Facility, and seasonal availability of cooperator staff to perform the releases, the number of wasps released at a given site often deviates significantly from this ideal; fortunately, the deviation is often that the site receives more than the suggested minimum number of insects.

Table 2.1. Releases of Female Parasitoids, by US State and County, 2009

State	County	<i>Spathius</i>	<i>Tetrastichus</i>	<i>Oobius</i>
IN	Orange/Orleans	0	750	0
MD	Prince George's	3000	2330	0
MI	Ingham	3150	500	0
OH	Franklin	3450	550	0
Total		9600	4130	0

Table 2.2. Releases of Female Parasitoids, by US State and County, 2010

State	County	<i>Spathius</i>	<i>Tetrastichus</i>	<i>Oobius</i>
IL	Cook	6614	14790	489
IN	Orange/Orleans	4264	10374	676
KY	Jefferson	3649	6893	0
KY	Shelby	534	1323	0
MD	Prince George's	11612	21645	678
MI	Delta	0	4470	1117
MI	Ingham	4796	13103	2087
MI	Ionia	3171	3263	0
MI	Tuscola	3171	3263	0
MN	Houston	910	1653	0
OH	Franklin	3863	4912	405
WV	Fayette	5229	8938	0
WV	Morgan	4138	6943	0
WV	Roane	2286	4140	0
Total		54237	105710	5452

Table 2.3. Releases of Female Parasitoids, by US State and County, 2011

State	County	<i>Spathius</i>	<i>Tetrastichus</i>	<i>Oobius</i>
IL	Cook	4230	8354	2101
IL	Lake	2100	2510	0
IN	Floyd	300	826	40
KY	Frankfort	2300	3398	0
KY	Fayette	6000	6857	0
KY	Jefferson	400	1026	782
KY	Kenton	1160	2644	639
KY	Shelby	1746	2727	394
MD	Alleghany	3400	4765	220
MD	Anne Arundel	1239	1382	175
MD	Howard	1666	3307	185
MD	Prince George's	12705	23247	2878
MI	Bay	400	400	200
MI	Delta	3285	6643	1231
MI	Houghton	8205	14574	1749
MI	Ingham	6204	13865	3612
MI	Ionia	6000	6039	1500
MI	Leelanau	800	1325	0
MI	Tuscola	6000	6000	1500
MN	Hennepin	5630	12316	3448
MN	Houston	400	723	200
NY	Cattaraugus	2400	2400	0
NY	Ulster	2800	2800	0
OH	Montgomery	1200	1300	200
PA	Allegheny	2800	4034	1733
PA	Union	1200	2081	700

VA	Loudoun	229	654	401
WI	Ozaukee	1800	2701	1389
WV	Berkley	2258	4437	999
WV	Calhoun	400	490	0
WV	Gilmer	3000	3286	0
Total		92257	147111	26276

A modification to the release strategy was made in the early months of 2013: *Spathius agrili* is no longer being released to areas north of 40°N latitude in North America, while releases will continue south of this line (USDA-APHIS 2013). This decision was based on a paucity of recovery data from those areas despite success at recovery of the other parasitoids (Duan et al. 2013), as well as more recent climate matching information. Small-scale research projects will continue in some areas to further understand the best practices for releasing *S. agrili*.

Future years will see a targeted, two-fold approach: one, new sites will be identified and approved for release based on suitability (ash density, EAB population size), spread of EAB into new areas, and parasitoid availability at the Rearing Facility; and two, sites at which releases have previously occurred will no longer be provided additional parasitoid material and will switch into recovery, establishment, and impact mode.

2.1.4 US recovery data

The federal EAB Program in the United States is interested in documenting overwintering and establishment of the parasitoids as first steps toward determining the efficacy of these biological control agents. Both processes are documented by active recovery of parasitoid life stages from in or around the release sites: overwintering is the recovery of live parasitoid life stages in the early spring following a release the previous summer or fall, whereas establishment is the recovery of parasitoid life stages in the late summer or early autumn one full year after releases (e.g. the live parasitoids found cannot be the immediate offspring of those insects released, and thus demonstrate independent reproduction in the field).

Recovery of the three parasitoids has occurred at sites across the US (see Table 2.4) but is only now beginning to reach the operational phase. The past two years have been spent in development of techniques for parasitoid recovery. The current best practices include a combination of yellow pan traps (filled with propylene glycol), “sentinel logs” created in the laboratory by exposing adult EAB to ash bolts and collecting the eggs deposited (these are then secured to ash trees in the field to allow parasitism by, and thus recovery, of *Oobius*), and debarking of entire trees harvested from release areas to find parasitized larvae. Of these methods, yellow pan traps are arguably the least labor intensive, but they have thus far failed to recover *Oobius*, necessitating the sentinel log strategy, which depends on having at least a limited laboratory space to rear the adults and allow them to oviposit. Debarking of trees, while highly laborious, provides the most complete data on parasitism rate and brood size in the field. Recoveries of both species of larval parasitoids have occurred at the same site in some cases, suggesting (see section 2.1.3) that they can coexist, at least at their current, low population density.

Finally, a male-produced pheromone blend emitted by *S. agrili* has been elucidated in the laboratory, and successfully tested in field cages (Cossé et al. 2012). Use of this technique to trap *S. agrili* in the field, for recovery and establishment studies, is undergoing further research and field testing and may become available in the future. Impact studies, which investigate actual changes

in the health of ash trees following parasitoid release and involving control sites where ash health is monitored in the absence of parasitoid releases, are ongoing in the US but are at least two years from completion.

Table 2.4. Sites of Parasitoid Recovery in the US, by State and Species

State	<i>Spathius</i>	<i>Tetrastichus</i>	<i>Oobius</i>
MI	X	X	X
MD	X	X	X
IL	X	X	
IN		X	
NY	X	X	
OH	X	X	X
PA	X		X

Recently, a significant milestone was reached during monitoring of the parasitoid populations at the original release sites across Michigan in the United States. It has been now shown at a suite of sites that *Tetrastichus planipennis* is capable of both establishing a population, increasing in parasitism rate on EAB over time, and also undergoing significant spread to surrounding areas (Duan et al. 2013). Projects are underway in other US states to examine the population dynamics of the parasitoids in the same way.

2.1.5 Mass-rearing facility – Methods development and process improvements

The process for rearing the EAB is the critical limiting factor in parasitoid production, and requires the most time and monetary investment – infested trees must be harvested from the field, sectioned, and held in rearing tubes to produce adults; a greenhouse must be maintained and kept pest-free to supply adequate quantity of high-quality ash foliage for more than half the year when outdoor sources are not available, and bark-stripping up to one thousand trees per year to collect larvae for use as hosts for the larval parasitoids. Thus, a major undertaking at the US Rearing Facility has been to investigate questions about the parasitoids and host EAB, with the overarching goal of facilitating increased production of quality insect material for release into the environment. Studies of the parasitoids are also being pursued that aim to understand their responses to climate, as they are being released over a vast geographic area, and there is a lack of thorough understanding of when it is best to release them in each specific location.

Host quality is often a critical factor in the decision-making process of female parasitoids (Charnov 1982; Godfray 1994; King 1987) and thus, improving host quality is of enormous importance to the mass-rearing operation. We have initiated two primary pathways of investigation toward improving host quality: provisioning of adult EAB with high-quality foliage and adjusting sex ratio and group size to maximize female fecundity to provide eggs for rearing *Oobius*, and developing techniques for rearing abundant, high-quality, EAB larvae for the larval parasitoids.

The first pathway, providing the adult EAB with optimal conditions, has focused on group rearing methods to increase efficiency in egg production while decreasing the labor costs involved. Novel strategies to acquire host material have also been pursued, such as contracting with southern

states in the US to pay for ash foliage to be sent to the Rearing Facility for winter use. Greenhouse-grown ash foliage does not fully mature, and provides a non-optimal nutrient profile for adult EAB, reducing fecundity and lowering the quality of egg produced, which in turn reduces the success of the egg parasitoid.

The second pathway, producing abundant, high-quality EAB larvae for use as larval parasitoid hosts, has yielded major increases in production efficiency. A major series of collaborations between the Facility and ARS has resulted in the development of a lab-based method to rear EAB larvae and adults; the only inputs needed from the field remain ash bolts and foliage, and this is unlikely to change in the future, as a practical and economical artificial diet for larval or adult EAB has remained utterly elusive. Perhaps the most important fruit of the research has been the development of a system where small ash bolts with specific numbers of EAB eggs implanted upon them can be used to produce predictable quantities of parasitoids. Testing has begun on a system to send these bolts to the field, to be hung on infested ash trees, before the emergence of the adult parasitoids. This has saved labor costs of counting and shipping the adult wasps, has reduced shipping mortality of the insects, and allows the insects to emerge naturally in the field, which is hypothesized to increase synchronization success.

Open questions remain, however. Extensive field testing of the new release methods will be undertaken in 2013 and monitoring will require at least 1-2 years after beginning releases. The results of the climate simulations, in conjunction with research collaborators at laboratories around the US, will likely take at least the next year to complete, and field operations may need to be modified afterwards to update best practices. A complete understanding is also lacking of the role of native parasitoids in the long-term ecological cycle of EAB infestation and natural enemy response.

2.2 Native parasitoids and predators of EAB

Surveys in southeastern Michigan indicated that parasitism by native parasitoids of EAB populations amounted to less than 1% (Liu et al. 2003). Five species of hymenopteran parasitoids were reared from different EAB life stages collected in Pennsylvania with a total parasitism rate of 3.6% (Duan et al. 2009). In spite of having examined large numbers of eggs of EAB, no egg parasitoids have been encountered in Michigan or Pennsylvania populations (Liu et al. 2003, Bauer et al. 2007, Duan et al. 2009). Natural enemies of *Agrilus* spp. related to the pest or parasitoids attacking populations of EAB in North America are being sought for an augmentative/inundative biocontrol strategy (Cappaert and McCullough 2009, Duan et al. 2009, Lyons 2010, Kula et al. 2010). These natural enemies could then be mass reared/cultured and introduced into sites with EAB populations.

Preliminary surveys for native parasitoids of EAB in Michigan encountered extremely low levels of *Atanycolus hicoriae* Shenefelt and *A. simplex* (Cresson) (Hymenoptera: Braconidae) that were associated with EAB larvae (Liu et al. 2003). A new species in the same genus, *A. cappaerti* Marsh and Strazanac, was subsequently described from EAB in Michigan (Marsh et al. 2009). Two additional species, *A. tranquebaricae* Shenefelt and *A. nigropyga* Shenefelt were also reported from EAB-infested ash bolts in Michigan (Cappaert and McCullough 2009). Three species, *A. cappaerti*, *A. hicoriae* and *A. longicauda* Shenefelt (identified by P. Marsh, United States National Museum, retired), were reared from EAB-infested logs from southwestern Ontario (Lyons 2010). Two of these species, *A. cappaerti* and *A. hicoriae*, plus an additional species, *A. disputabilis* (Cresson), were also collected from *Agrilus anxius* infested white birch logs in the vicinity of Sudbury, Ontario. In studies sites near Lansing MI, parasitism of EAB by *Atanycolus* spp. increased from nine individuals to 774 individuals in one year (Duan et al. 2012a). About 93% of these parasitoids were *A. cappaerti* and the remainder were *A. hicoriae* (5%), *A. tranquebaricae* (1%), and *A. disputabilis* (<1%). Cappaert and McCullough (2009) began investigations into the biology and biocontrol potential of *A. cappaerti*. Observed parasitism rates ranged from 9 to 71% in two

sites over two years suggesting that this species might be an effective biological control agent for EAB. Those authors observed a bivoltine life cycle for this idiobiont ectoparasitoid with a generation time of less than 30 days. Male:female sex ratio of the parasitoids was 1:6 with similar median adult emergence dates for the sexes in late May. Duan et al. (2012a) reported a very different male:female sex ratio for *A. cappaerti* of 1:0.6. Males and females of *Atanycolus* spp. reared from log bolts collected in Ontario were extremely long lived with males and females living for an average of more than 100 days at 21°C (Lyons 2010). Recent studies indicates that *Atanycolus* spp. are able to attack EAB under much thicker bark than can the introduced *T. planipennis* (Abell et al. in press).

Leluthia astigma (Ashmead) (Hymenoptera: Braconidae), which was positively associated with EAB cadavers, was reported as the most abundant parasitoid attacking EAB in Delaware Co., Ohio with a parasitism rate of 2.1% (Kula et al. 2010). The species is a solitary idiobiont ectoparasitoid that is broadly distributed in North America from California and Guadalajara Mexico to North Carolina and Quebec (Kula et al. 2010). Adults that emerged from cocoons successfully produced a new generation on EAB larvae inserted into ash sticks (Kula et al. 2010). Two specimens of this species were collected in Ontario (Lyons 2010). One specimen was collected from green ash infested with EAB near Windsor and the other specimen was collected from red oak infested by *Agrilus bilineatus* near Midland. Although reported from Quebec (Smith et al. 1979) these are the first specimens collected from elsewhere in Canada.

Spathius floridanus Ashmead (Hymenoptera: Braconidae) (= *S. simillimus* Ashmead; synonymised by Marsh and Strazanac 2009) was one of the species encountered by Liu et al. (2003) and Duan et al. (2012b) during surveys for native parasitoids of EAB in Michigan. Known hosts for *S. floridanus* include *Agrilus anxius* Gory, *A. bilineatus* (Weber), *Chrysobothris femorata* (Oliv.) (Coleoptera: Buprestidae); *Magdalis olya* (Herbst) (Coleoptera: Curculionidae); *Phymates aereum* (Newman) and *Xylotrechus colonus* (F.) (Coleoptera: Cerambycidae). The species has been reported previously from New Brunswick to Ontario in Canada and from Wisconsin to Texas (Marsh and Strazanac 2009). Marsh and Strazanac (2009) consider this species the most promising native *Spathius* as a candidate for biological control of EAB. Fifteen specimens of this parasitoid were reared from red oak infested with *A. bilineatus* near Midland Ontario, but the species has not been reared from EAB in Ontario (Lyons 2010). *Spathius* spp. are gregarious (Marsh and Strazanac 2009) and thus this record may represent a small number of oviposition events. Another undescribed species of *Spathius* has recently been recorded from EAB in Michigan (Marsh and Strazanac 2009).

Balcha indica (Mani & Kaul) (Hymenoptera: Eupelmidae) was one of the parasitoids encountered during surveys for native parasitoids of EAB in Michigan (Liu et al. 2003, Duan et al. 2012a) and Pennsylvania (Duan et al. 2009). *Balcha indica* is itself an alien species, which is native to southeastern Asia (collection records from Burma, India, Thailand, and Vietnam) and probably arrived in North America on some host other than EAB because its discovery in Virginia in 1994 pre-dates the estimated arrival of EAB in that area (Gibson 2005). In North America only females of *B. indica* are known, although males are known to occur in Asia (Gibson 2005). This parasitoid was the most abundant species reared from EAB in Pennsylvania and successfully reproduced parthenogenetically on EAB in the laboratory (Duan et al. 2009). The life history and stages of this solitary ectoparasitoid was described by Duan et al. (2011a). The wasps parasitize larvae, prepupae and pupae of the host. Females begin to oviposit during the first week after emergence with peak emergence occurring several weeks later. Eggs are attached to the host by sticky strands. Mean fecundity was 35.8 eggs. First-instar larvae have sclerotized head capsules and mandibles while later instars do not. It takes about 3 months to complete a generation at 25°C. The long univoltine generation time for *B. indica* may hamper effective rearing methods and thus limit its utility as an augmentative biological control agent against EAB (Duan et al. 2011b). Small numbers of *B. indica* have routinely been collected from EAB infested logs in Ontario (Lyons 2010).

A single specimen of *Metapelma spectabile* Westwood (Hymenoptera: Eupelmidae) was reared from an EAB-infested ash bolt collected near Windsor, Ontario. This species has been reported from *Agrilus angelicus* Horn but there is no previous record from Canada (Burks 1979).

Green ash log bolts collected from a site in southwestern Ontario in the later stages of an EAB outbreak produced unprecedented numbers of *Phasgonophora sulcata* Westwood (Hymenoptera: Chalcididae) (Lyons 2010). Of the 215 insects reared from these bolts 146 were *A. planipennis* and 54 were *P. sulcata*. At a nearby location eight *P. sulcata* emerging from bolts were greatly outnumbered by 648 emerging EAB adults, suggesting a parasitism rate of only about 1.2%. Apparently parasitism rates vary spatially and probably temporally as well. In 2007, sticky-band traps were placed in the woodlot in Essex Co. from which the high number of *P. sulcata* had been obtained. Six hundred EAB adults and 407 adults of *P. sulcata* were collected from the traps suggesting a parasitism rate of 40.7% (Lyons 2010). In the laboratory, mean emergence of *P. sulcata* was after the mean emergence of EAB which corresponds with the observed late flight period of the parasitoid relative to the host observed in the field in Ontario (Lyons 2010). The flight period of the parasitoid seems to be synchronized with the egg laying period of EAB and supports the observation by Haack et al. (1981) that the parasitoid may lay eggs near the host's eggs. Mean longevities for *P. sulcata* male and female adults were approximately 25 and 39 days, respectively (Lyons 2010). This is one of the parasitoid species reared from EAB in Michigan (Liu et al. 2003) and has been reported from *Agrilus bilineatus* (Weber) (Haack et al. 1981), *A. anxius* Gory (Akers and Nielsen 1990), and *A. granulatus liragus* Barter and Brown (Barter 1965).

Other species of hymenopteran parasitoids that have been reared from EAB but have not been encountered in Ontario include: *Eupelmus pini* Taylor (Eupelmidae); three ichneumonids, *Dolichomitus vitticrus* Townes, *Orthizema* sp., and *Cubocephalus* sp. (Duan et al. 2009); and unknown species of *Dolichomitus* (Ichneumonidae) and *Eurytoma* (Eurytomidae) (Duan et al. 2012a). Duan et al. (2009) successfully reared the thelytokous parthenogenetic *E. pini* on EAB larvae, prepupae and pupae inserted into ash sticks in the laboratory.

Apart from native parasitoids, other North American organisms have made a limited transition to preying on EAB populations. A few predatory coleoptera (e.g., *Enoclerus* sp. (Cleridae), *Catogenus rufus* (F.) (Passandridae) and *Tenebriodes* sp. (Trogossitae)) have been collected feeding on EAB life stages under the bark of host trees (Liu et al. 2003). According to Cappaert et al. (2005), woodpecker predation is probably the most important source of mortality in EAB populations in Michigan and has accounted for 9 to 95% mortality. Next to host tree defences, woodpecker predation was the second highest source of mortality for experimental populations of EAB (Duan et al. 2010). Lindell et al. (2008) have observed hairy, downy and red-bellied woodpeckers preying on EAB and recommended the maintenance of conditions (e.g., nest sites) that attract woodpeckers. Ironically, woodpeckers have little impact on EAB populations in Asia (Duan et al. 2012a). The predatory wasp, *Cerceris fumipennis* Say (Hymenoptera: Crabronidae), has been shown to collect EAB adults to provision its nest. Although the impact of the wasp on EAB populations is not significant, this species may prove to be a useful biosurveillance tool for detecting low density EAB populations (Marshall et al. 2005; Careless et al. 2009).

2.3 Biological control using entomopathogenic microorganisms

Lack of predators, apparent lower host tree resistance, food availability and favourable climatic conditions, all contribute to the spread of invasive exotic insects (Hajek 2009, Tanis and McCullough 2012). The emerald ash borer (*Agrilus planipennis*) (EAB) discovered in North America in 2002 is still difficult to detect at the endemic level (Crook and Mastro 2010). Eradication is no longer an option to consider and the current strategy is to slow the spread of the EAB using various tools (Marchant 2007). However, effective measures are needed to slow the spread of the EAB, to contain isolated infestations and to control its populations at or below a tolerance threshold for survival of ash trees (Liu and Bauer 2008a). Apart from regulations and tree cutting activities,

some insecticides are being used against EAB in US and Canada but their use is limited, expensive and difficult to apply on a large scale. The interest in biological control with entomopathogens, as part of an integrated pest management approach, is increasing. Ash trees have been largely planted in cities and in these areas the use of microbial control agents is thus more amenable from an environmental point of view (Hajek and Bauer 2007). Entomopathogenic microorganisms are natural and could offer a partial solution, not to eradicate EAB but to reduce the impact of the EAB in North America.

Different entomopathogenic microorganisms have been considered for use against EAB but the use of fungi has received much more attention than other organisms such as nematodes, microsporidia, or bacteria (Hajek and Bauer 2007). Scientific research behind the development of biological control of the EAB with entomopathogens is relatively limited. The following paragraphs will present the main studies done by different research teams to improve the knowledge about the use of entomopathogens against EAB.

2.3.1 Biological control with entomopathogenic fungi

Entomopathogenic fungi such as *Beauveria*, *Metarhizium*, *Isaria* and *Paecilomyces* are only a few of the genera of fungi that are known to be pathogenic against a large number of insects (Humber 2012). Also, entomopathogenic fungi have been used in management of many defoliating insects in agriculture like the Colorado potato beetle, the European corn-borer, whiteflies, grasshopper, locust, whiteflies, borers, aphids, weevils (Inglis et al. 2001, Vandenberg 2007a).

Among the wood-boring insects, encouraging results have been obtained for the Japanese pine sawyer (*Monochamus alternatus* Hope), the Asian longhorn beetle (*Anoplophora glabripennis*(Motschulsky)) using fungal bands, the emerald ash borer (*A. planipennis*), the European spruce bark beetle (*Ips typographus* (L.)) where horizontal transmission of *B. bassiana* among adults was demonstrated (Hajek and Bauer 2007, Kreutz et al. 2004).

Some commercial products based on micro-organisms are already available in Canada and US including BotaniGard®, Met52®, Naturalis L®, and Mycotrol EL® (Kabaluk et al. 2010).

2.3.2 Mode of action of entomopathogenic fungi

Compared with other insect pathogens, fungi can invade live insects through the cuticle and proliferate in their body cavity (Liu and Bauer 2006). The conidia of an appropriate isolate will germinate in contact with an insect cuticle, produce enzymes, invade the insect body, produce toxins and eventually kill the insect (Inglis et al. 2001). If environmental conditions are appropriate, the fungi will emerge from the insect body and produce conidia which may also be dispersed horizontally to other susceptible insects by wind or by direct contact. Entomopathogenic fungi can cause infections in all life stage but not all stages in insect's life cycle are equally susceptible to infection (Inglis et al. 2001; Hajek and St. Leger 1994). All life stages of EAB, except the eggs were susceptible to fungal infection under field conditions (Bauer et al. 2004c). Because EAB adults are found to be active on the tree canopy foliage or moving on the trunk, they represent an easier target than larvae. Adults could also come in contact with conidia sprayed on ash trunks as they emerge, while walking or ovipositing, and on foliage while feeding (Liu and Bauer 2006, 2008a, b). However, larvae are also susceptible because the high humidity under the bark provides ideal conditions for fungal spore germination on insect cuticle (Liu and Bauer 2006).

2.3.3 Most significant results of entomopathogen research for EAB

When the EAB was discovered in North America in 2002, the control options available were limited. According to Bauer et al. (2004c), the only way to control the EAB at that time was the identification and destruction of infested trees.

In 2003 and 2004, Bauer et al. (2004b) did a survey on entomopathogenic fungi found in over 6000 larvae collected in a Michigan woodlot. Surprisingly, 2% of immature EAB were infected with five species of fungi: *Beauveria bassiana* (24 isolates), *Isaria* (= *Paecilomyces*) *farinosus* (30 isolates), *I.* (= *Paecilomyces*) *fumosoroseus* (7 isolates), *Verticillium lecanii* (36 isolates) and *Metarhizium anisopliae* (2 isolates).

Bauer and colleagues (2006) were the first to run experiments to document if *Bacillus thuringiensis* (Bt) and *Beauveria bassiana* could be used against the EAB based on the safety records of these microorganisms and their compatibility with biocontrol. Four registered Bt strains were tested against the EAB and some strains were found to be toxic at high concentrations. Rates of 4 to 12 times the maximum labelled rates were required to achieve 66 to 98% mortality 6 days after treatments (Bauer et al. 2006). It was concluded that further research was needed to identify the active toxin and eventually develop an efficient tool for aerial spraying. Bauer and Londono (2010) screened 25 Bt strains having a known coleopteran toxicity including SDS-502 and Bt-Fc. The Bt SDS-502 was the most toxic. This Bt expresses the Cry8Da protein toxin which has shown activity in some coleoptera. Mortality of EAB adults exposed to Bt SDS-502 occurred within 96 hours of feeding on sprayed leaves and total mortality was similar for both Bt strains.

In 1995, the entomopathogenic fungus *B. bassiana* var. GHA was registered under the trade mark BotaniGard® against forest and shade tree pests. Bauer et al. (2004b) were the first to try BotaniGard® against the EAB with the objective of identifying a product for possible use in an aerial spray program.

Under laboratory conditions (Petri dish and cut foliage), Liu and Bauer (2006) exposed EAB adults to different isolates of *B. bassiana* and *M. anisopliae* to document their virulence (*B. bassiana* isolates: ARSEF 6393, 7152 and GHA and two *M. anisopliae* isolates: ARSEF 7180 and 7234). EAB were immersed in one of the two doses tested: 10^6 or 10^7 conidia/ml. Results showed that EAB adults were susceptible to *B. bassiana* and *M. anisopliae* when treated by direct immersion as well as foliar exposure. The cumulative mortality at 6 d after treatment ranged from 80% to 97% and 97% to 100% for both concentration of 10^6 and 10^7 conidia/ml respectively. The average time-to-death values were lowest for *B. bassiana* GHA with 4.6 and 4.2 d at 10^6 and 10^7 conidia/ml respectively.

Later, in 2003, under greenhouse and field conditions the virulence, the lethal and sublethal effects of *B. bassiana* strain GHA (BotaniGard ES) were studied on EAB adults and larvae by Liu and Bauer (2008a) with topical spray and fungal band treatments. Results from greenhouse and field studies demonstrated that *B. bassiana* strain GHA has a good potential as a management tool for suppressing EAB population densities. Early summer pre-emergent trunk spray with *B. bassiana* infected and killed EAB adults, which became infected under the bark before emergence, possibly through entry of fungus conidia into bark splits, during emergence when chewing through fungal treated bark or after emergence while walking and ovipositing. Results also suggest a sublethal effect including shortened adult longevity and a prolonged larval development period. Finally, in July 2003, a fungal band impregnated with sporulating fungal culture *B. bassiana* strain GHA was applied to uninfested ash tree trunks in the field targeting EAB caged adults. The advantage to use a fungal band was the one month persistence of virulent conidia. As opposed to the results obtained with *A. glabripennis*, Liu and Bauer (2008a) mentioned that this technique may not be efficient against the EAB because the adults fly more than they walk.

In 2004-2005, Liu and Bauer (2008b) evaluated the effect of *B. bassiana* strain GHA applied with a back pack CO₂ sprayer on ash tree foliage or tree trunk of newly colonized and well established populations of EAB. Trees treated with *B. bassiana* GHA contained 41% fewer younger larvae than did control trees and 20% of the larvae on trees from treated areas were infected after 14 days of laboratory incubation. *B. bassiana* treated trees suffered less crown dieback than did the controls with an increase in dieback of 34% for treated compared with an increase of 57% for the control

trees. Fungal treatments significantly reduced crown dieback. In trees treated with the GHA strain, the larval density was almost half that in the control with an average density of 55 and 103 larvae/m², respectively. The average emerged adult density was 57 adults/m² for control trees whereas only 21 adults emerged in fungal treated trees. Moreover, Liu and Bauer (2008b) found a good conidial persistence in the field. After the 2-day exposure period to treated foliage, adult mortality ranged from 78% for those exposed to foliage collected 24 h following fungal application to 100% on foliage collected 2 h after spray application. Adults exposed to treated foliage had a shorter lifespan compared with those exposed to control foliage. Also the lowest time to death was observed for adults exposed to foliage collected 2 h after application. Adult mortality occurred mainly between 4 and 7 days after exposure to *B. bassiana* treated foliage (Liu and Bauer 2008b). Liu and Bauer (2008b) have shown improved field efficacy of GHA when sprayed to trunks prior adult emergence. These authors found infection rates ranging from 58.5-83% in EAB adults in the field. They also observed the infection rate in larvae correlated to larval density on the field. Consequently, they concluded that there was horizontal transmission of the disease.

Few trials combining entomopathogen fungi and chemical pesticides have been done. Vandenberg et al. (2007b, 2008) evaluated the impact of *B. bassiana* used alone or in combination with imidacloprid in infested trees in a nursery. Mortality of EAB adults exposed to leaves treated with both imidacloprid and the fungus was equal to, or greater than, that of EAB exposed to leaves treated with fungus alone.

To be an effective control tool against the invasive EAB, the fungus *B. bassiana* GHA needs to be applied during an appropriate treatment window. The objective is to target the emerging adults in early summer, during the maturation feeding period and before females lay eggs to maximize impact on EAB populations. Castrillo et al. (2010a) performed a study on deposition and persistence of *B. bassiana* under field conditions in a tree nursery. *B. bassiana* GHA was sprayed on ash boles and on tree canopy with a backpack sprayer and a hydraulic sprayer. The estimates of *B. bassiana* Colony Forming Unit (CFU) obtained after pressing leaflet surfaces presented higher counts after 7 days vs. 14 days on two tests out of three. Samples taken 28 days after the third spray still showed viable CFUs. After 14 days following canopy spray, the number of CFUs on the upper and lower side of foliage was finally similar. The reduction on the upper side foliage can be explained by the UV light exposure with consequent reduction in viability (Inglis et al. 1993, Moore et al. 1993) and wash-off by rainfall (James et al. 1995). The dense and overlapping canopy in ash trees could however provide shading to minimize UV damage to conidia on the upper leaf surface. Even though the level of inoculum was reduced to 2- to 5-fold less on leaves, mortality was still observed among EAB exposed to these leaves. Surprisingly CFUs were still obtained from ash leaves approximately 28 days after the last spray application in 2006. To follow the persistence of *B. bassiana* GHA isolate in natural environments after mass release, real-time PCR primers and probes were developed (Castrillo et al. 2008).

Castrillo et al. (2010a) have shown that even on bark, 14 days after spray, the mortality of EAB exposed 24 h to the sprayed bark was 40%. Persistence of conidia on bark showed a similar trend as with foliage. In contrast to leaflets, estimates for bark indicate that mortality remains comparable from 7 and 14 days after each spray. Better persistence on bark may likely be due to the presence of cracks and crevices on ash bark in which conidia get lodged during high pressure spray application. Data obtained by Castrillo et al. (2009a,b) suggest that EAB adults carry the fungus from one tree to another.

In the years following the EAB discovery in Michigan, field sampling of presumably indigenous fungal isolates pathogenic to EAB were performed. Between 2002 and 2006, *B. bassiana* was isolated from larvae and prepupae from infested ash tree felled in Michigan. Recently, they compared the virulence of representative isolates against EAB (Castrillo et al. 2010b), but they did not find any significant difference in mortality between indigenous strains and GHA. After 6 days following treatment, mean mortality among control EAB was 18.3 ± 3%. The estimated GHA

mortality at 3.6×10^6 conidia/ml, the same dosage used for EAB derived strains, was $90 \pm 12\%$. From their analysis, Castrillo et al. (2010b) concluded that soil serves as the primary source of fungal inocula that may be dispersed to tree trunks by rain splash or by air currents. Also, natural dispersal can occur through birds like woodpeckers when these forage on EAB-infested ash trees (Lindell et al. 2008). According to Castrillo et al. (2010b), dispersal of indigenous *B. bassiana* propagules, possibly by air current, rain splash, or infected insects, onto ash trunks limits widespread dispersal and heavy inocula build up that could trigger epizootics of the fungus. Male and female EAB feed on ash foliage through their adult stage, but females also spend considerable time in contact with ash bark during emergence, when they chew through the tree bark, and when mating and searching for oviposition sites during egg deposition (Liu and Bauer 2006, 2008 a, b).

The mode of inoculum uptake and level of inoculum present on ash trunk constrains infection prevalence in EAB populations (Castrillo et al. (2010b). The bark is a more accessible source for EAB infection and Castrillo et al. (2010b) suggest applying GHA prior to adult emergence since bark is a reservoir and GHA is virulent.

Indigenous pathogens have an important advantage over exotic natural enemies in that they are already adapted to the pest's habitat and pose lower risk of community disruption (Johny et al. 2012a). Johny et al. (2012a) looked for indigenous entomopathogens in south Ontario and most isolates were recovered from EAB pupae and larvae, with lesser numbers from EAB adults. The isolates were concentrated in two major groups, *Beauveria bassiana* and *Beauveria pseudobassiana*. All the EAB-recovered *Beauveria* spp. isolates bio-assayed were found to be pathogenic with variability in their virulence to EAB adults. Significant differences were observed in cumulative insect mortality between 4 and 14 days after treatments with four different conidial concentrations among the 8 isolates derived from EAB and the commercial GHA isolate. The isolate L49-1AA was about five times more virulent than GHA while LD20A and LB25C were the only isolates that were significantly less (about 3 times) than the commercial isolate. Isolate L49-1AA killed EAB adults faster than all other isolates with an MST of 6.16 days. However, it did not significantly differ from GHA (6.93 days) and B4B (7.4 days). Isolate L49-1AA had the lowest LC50 followed by GHA. An important aspect that was highlighted is related to the conidial production, where the highest conidia formation was observed for L49-1AA (1.38×10^8 conidia/insect). As mentioned by Johny et al. (2012a), to develop an autocontamination strategy, high production of conidia on cadavers could partially compensate for low survival due to temperature, moisture and UV radiation.

In a survey, Johny et al. (2012b) found six fungal strains belonging to *Isaria farinosa* (Holmsk) (formerly *Paecilomyces farinosus*) and a new *P. lilacinum* colonizing the EAB in southern Ontario. These pathogen isolates may have adapted to EAB in Ontario and are now part of the natural control agents in Canada, having been retrieved for two years.

Current field control strategies of applying GHA-based mycoinsecticide to the bark prior the borer emergence could prove viable since this strain (GHA) is virulent to EAB (Liu and Bauer 2006, 2008a,b) and also can persist for several weeks on ash bark (Castrillo et al. 2010a). However, early in the infestation cycle, EAB adults may be less likely to encounter fungal inocula on bark (Castrillo et al. 2010b). When adults first arrive in the infestation cycle they generally attack healthy trees along the upper trunk (unpublished data in Castrillo et al. 2010b). EAB feed on ash foliage throughout their life. Consequently bark spraying may be complemented with traps available in tree canopy to deliver the fungus via an autocontamination strategy. In 2010 and 2011, Lyons et al. (in press) modified an existing insect trap to allow the autocontamination of insects with entomopathogenic fungi. Field results were promising and demonstrated a season-long persistence of the inoculum and the recapture of EAB adults in sticky traps bearing spores.

Beauveria is a wide spectrum entomopathogen and its use could be limited by the negative side effect on useful insects. Dean et al. (2012) performed a study to determine susceptibility of

Spathius agrili and *Tetrastichus planipennisi* to *B. bassiana* in the laboratory. Interestingly, *T. planipennisi* had a very low or no susceptibility to *B. bassiana* GHA under laboratory conditions. All 88 EAB exposed to fungus-inoculated ash twigs died within 6 days. *S. agrili* was slightly more susceptible to *B. bassiana* with an average difference of 13% in mortality between treated and control groups. This may not be of substantial biological consequence as only 13 *S. agrili* died out of the 84 exposed.

A new microsporidia was isolated from the bronze birch borer by Kyei-Poku et al. (2008, 2011) and studies are ongoing to determine if the pathogen can infect EAB. New nematode species closely related to *Rhabditis* species were recovered from EAB cadavers in Ontario by Kyei-Poku et al. (2009). The isolate kill EAB larvae, *Tenebrio molitor* and *Zophobas morio* larvae within 2-4 days. This nematode is being investigated further for potential biological control of EAB.

In conclusion, entomopathogenic fungi can represent a complement to other biological, mechanical and legislative control measures to slow the spread of EAB. Direct application of efficient strains can reduce populations of EAB. Moreover, dissemination of the fungi by autocontamination may help to reach other adults while mating in the canopy. Entomopathogenic fungi represent a low cost and environmentally acceptable product. Besides direct mortality of EAB, the impact of entomopathogenic fungi on populations of EAB and reduction in tree mortality must be demonstrated.

3. Regulatory processes associated with the importation, the rearing, the release and the domestic movement of biological control agents of insect pests

In North America, the importation and release of entomophagous biological control agents requires that an applicant submits an application for permit to import and a petition. The petition should include all the elements outlined in the Regional Standard for Phytosanitary Measures (RSPM) 12: 2008. For the first release of non-indigenous phytophagous biological control agents, RSPM 7: 2008 guidelines should be used.

RSPM 7: 2008 and RSPM :12 2008 guidelines are intended to assist in drafting petitions for release of biological control agents. The petitions provide reviewers and regulators the information needed to assess the risk of non-indigenous introductions intended for biological control of insect pests. As the petition review process in Canada, U.S. and Mexico includes consultation with the two other NAPPO countries, these guidelines allow for a standardized petition format. The guidelines could also be used for biological control agents for other target pests (e.g. mites, nematodes, and molluscs) at the discretion of the National Plant Protection Organization.

NAPPO standards represent an important tool to harmonize the data required for the release of biological control agents in North America. All petitions submitted to the CFIA, to APHIS and to SAGARPA must conform to the standards set out in the NAPPO guidelines.

The following sections describe the specific regulatory processes related to the importation, rearing and release of biological control agents in each NAPPO country.

3.1 Regulatory processes in the United States

In the US, the biological control agents of plant pests and noxious weeds are regulated under the *Plant Protection Act* (2000). The *Plant Protection Act* provides a definition for biological control agents and recognition of their potential to control plant pests. The Plant Protection and Quarantine (PPQ), Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) administers the *Plant Protection Act*. This gives them the authority to regulate the importation, interstate movement and environmental release of biological control agents.

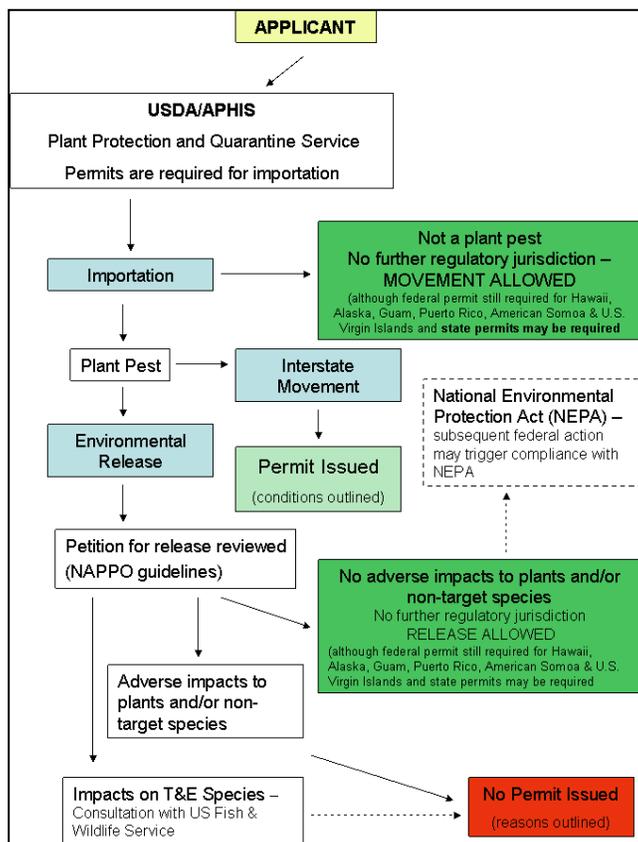
Separate permits, issued by the USDA-APHIS-PPQ, are required for importation, interstate movement, possession and/or release into the environment of biological control agents (APHIS 2012; University of Florida 2012). The PPQ Form 526 must be completed (APHIS 2012). The form is available online using the APHIS ePermit system (see http://www.aphis.usda.gov/permits/learn_epermits.shtml). The ePermit system allows the applicant to submit and track permit applications, receive permits, and apply for renewals and amendments online. The permitting process may take four to six months to complete. Subsequent approval for a permit may also be required by the individual states.

For biological control agents that are known not to be a plant pest, an import permit is required for entry into the US. The biological agents will be inspected by PPQ at an inspection station at the point of entry and then no further requirements will apply for the domestic movement of the agents (except for Hawaii, Alaska, Guam, Puerto Rico, American Samoa and U.S. Virgin Islands where federal permits are required). State permits may also be required.

For biological control agents that could be a plant pest, a permit is required for the importation as well as for the interstate movement, if applicable (conditions for movement will be outlined on the permit). Permits to import biological control agents from off-continent are issued to approved containment facilities to verify the identity and purity of the biological control agent and/or to gather scientific information that would be required to prepare a petition for release of the biological agent in the environment (Mason et al. 2005).

In cases where release in the environment is considered, a petition for release is prepared as per the NAPPO guidelines for review. If potential adverse impacts to plants and/or non-target species are identified, a permit will not be granted. In that case, the rationale for that decision will be provided to the applicant. If no adverse impacts to plants and/or non-target species are identified, release in the environment will be allowed. For detailed information on field release of approved EAB biological control agents, please consult the EAB Biological Control Release Guidelines at http://www.aphis.usda.gov/plant_health/plant_pest_info/emerald_ash_b/downloads/EAB-FieldRelease-Guidelines.pdf

Figure 3.1. Regulatory processes associated with the importation and release of biological control agents of insect pests in the United States (from Mason et al. 2005) USDA= United States Department of Agriculture; APHIS= Animal and Plant Health Inspection Service; T&E=Threatened and Endangered



3.2 Regulatory processes in Canada

In Canada, biological control agents are regulated through the *Plant Protection Act* (PPA), administered by the Canadian Food Inspection Agency (CFIA) (Mason et al. 2005; De Clerck-Floate et al. 2006). The PPA was enacted to prevent the introduction and spread of exotic plant pests. Organisms that are directly injurious to plants and predators and parasites/parasitoids of phytophagous organisms are considered plant pests. Thus the importation and release of entomophagous arthropods for biological control are regulated under the PPA (De Clerck-Floate et al. 2006).

In order to be allowed to import a biological control agent in Canada, importers must submit an application for a Plant Protection Import Permit (http://www.inspection.gc.ca/DAM/DAM-plants-vegetaux/STAGING/text-texte/c5256_1331652913719_eng.pdf) with an information package for the petition to the CFIA Permit Office. Information is requested on the proposed action, on the target pest, on the biological control agent, on the environmental and economic impacts of the proposed release and on post-release monitoring. The applicant will be notified by the Permit Office if the documentation provided is insufficient. The process is the same no matter if the purpose of the import is for classical biological control (one time introduction that is expected to be self-sustaining) and for commercial agents that are inundatively introduced at regular intervals to control pests in usually protected environments (e.g., greenhouses).

As per De Clerck-Floate et al. (2006), 'petitioning applies whenever there is a planned introduction of a species, subspecies or even a population of a candidate species, particularly if introduced from

a geographic area/population than what may have been previously introduced or tested for introduction’.

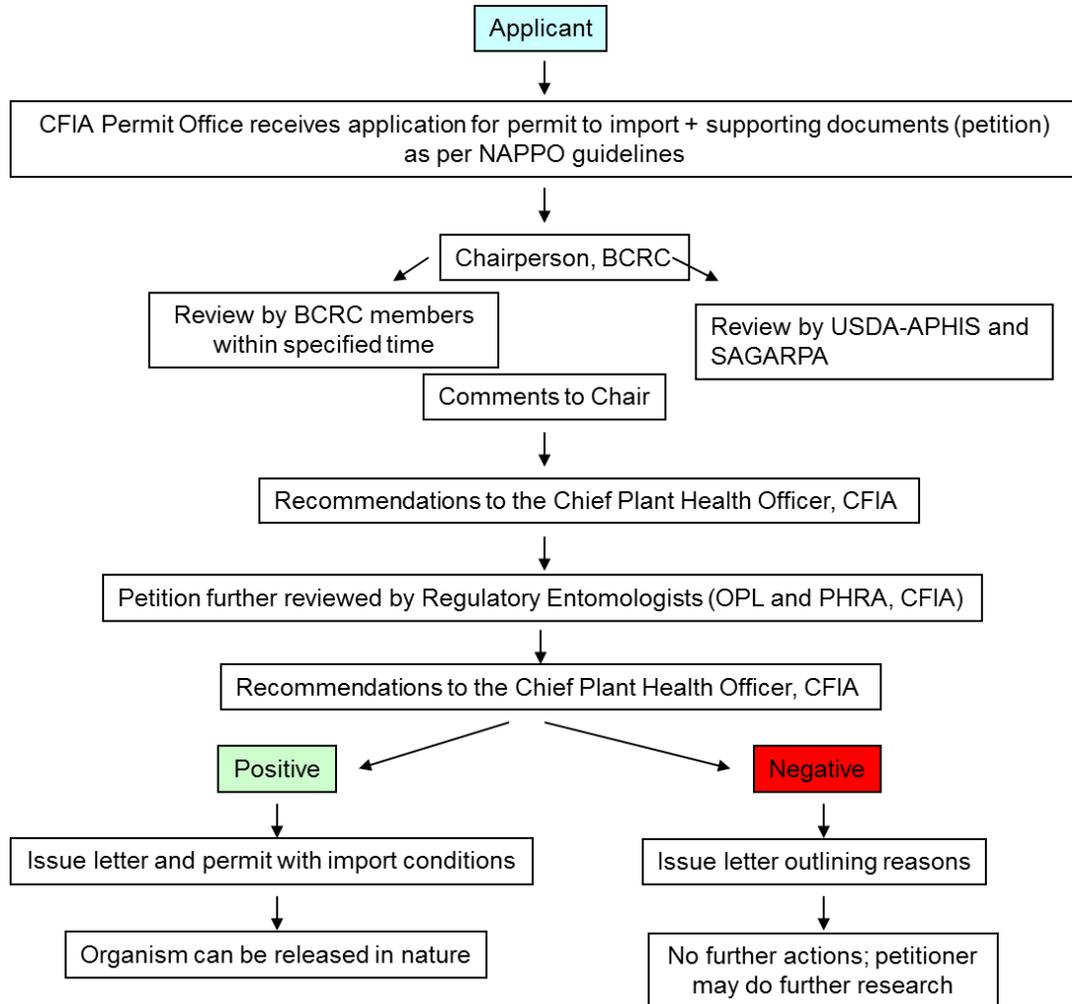
An import permit is required for all importations of commercial biocontrol agents but a petition is not always required (De Clerck-Floate et al. 2006). In cases where a petition is required and provided with the import permit application, it will be forwarded to the Chairperson of the Biocontrol Review Committee (BCRC) of Agriculture and Agri-Food Canada (AAFC) for review. The BCRC may include taxonomists, ecologists, scientists, specialists in federal and provincial governments and Canadian universities, consultants and representatives from the Pest Management Regulatory Agency (PMRA) of Health Canada. The petition will also be reviewed by representatives from the USDA-APHIS and from the Mexican Secretary of Agriculture, Livestock, Rural Development, Fish and Food (SAGARPA) and their comments will be taken into consideration before a final decision is made (Mason et al. 2005; De Clerck-Floate 2006).

Once the petition is reviewed, the BCRC provides the recommendation to the Chief Plant Health Officer, Director of CFIA’s Plant Biosecurity and Forestry Division. The Chief Plant Health Officer makes the final decision and a letter is sent to the applicant advising him of the decision (Approved or Not Approved for release in Canada). If the release is approved, an import permit will be issued. The Automated Import Reference System (AIRS) will also be updated to reflect the decision made.

Once approved for release, the biological control agent can be released anywhere in Canada. Provincial representatives are part of the review committee and provincial officials are notified prior to the release, especially the province where the release is to occur.

Organisms could be allowed to be imported into a CFIA approved Canadian containment facility for receipt and containment of the organisms. This is typically done to allow for research on candidate biological control agents prior to petition for their release in the country. If the release is eventually approved, the import permit is then amended to reflect that decision.

Figure 3.2. Canadian review process for import and release of new entomophagous biological control organisms. BCRC= Biological Control Review Committee; CFIA= Canadian Food Inspection Agency; NAPPO= North American Plant Protection Organization; OPL= Ottawa Plant Laboratories, CFIA; PHRA=Plant Health Risk Assessment Unit, CFIA; SAGARPA = Secretary of Agriculture, Livestock, Rural Development, Fish and Food of Mexico (modified from Mason et al. 2005)



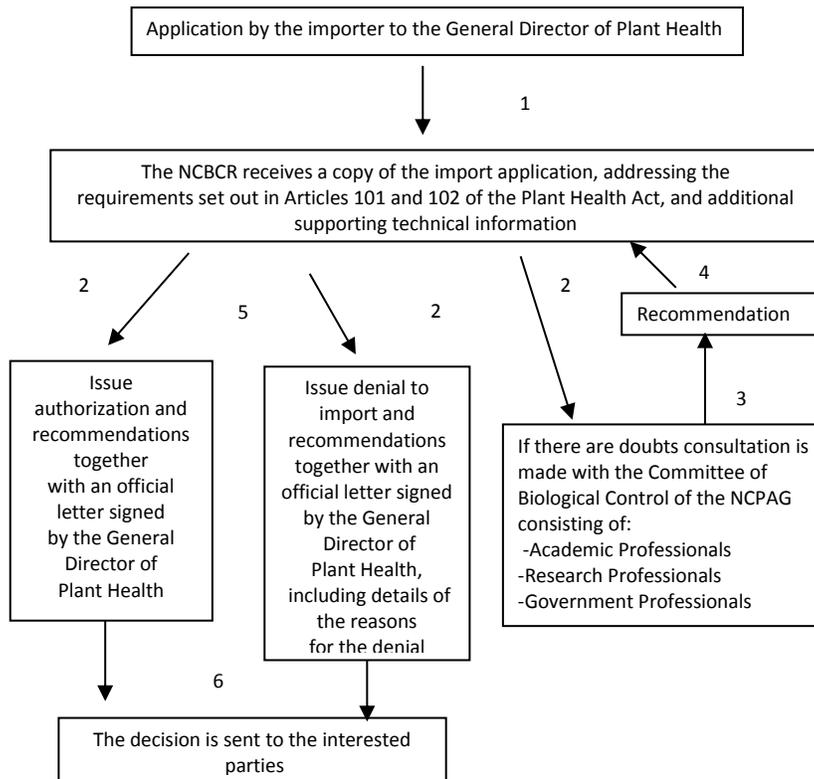
3.3 Regulatory processes in Mexico

In Mexico, SAGARPA is responsible for the administration and enforcement of the Plant Health Act of the Mexican United States, which regulates the importation of biological control agents in Mexico (SARH 1980 in Mason et al. 2005).

For the importation of biological control agents, importers must submit an import application to the General Director of Plant Health within SAGARPA. A copy of the application will be sent to the National Center of Biological Control Reference (NCBCR) for review. A petition, meeting RSPM 12: 2008 requirements must be provided with the import application.

If the biological control agent is not known to the NCBCR, the import application and the petition will be reviewed by the Committee of Biological Control of the National Consultative Phytosanitary Advisory Group (NCPAG), which includes Academic, Research and Government Professionals. Once the review completed, the committee will provide a recommendation to the NCBCR (Mason et al. 2005).

Figure 3.3. Mexican review process for import and release of biological control organisms. NCBCR= National Center of Biological Control Reference; NCPAG= National Consultative Phytosanitary Advisory Group (from Mason et al. 2005).



If the biological control agent is known to the NCBCR or once it receives a recommendation from the NCPAG for a new biological control agent, the NCBCR will either issue an official authorization or denial letter, signed by the Director General of Plant Health, to the importer. The recommendations and, if applicable, the reasons for the denial, will also be provided. A one year import permit will be issued. Among the import requirements that will be stated on the import permit, the biological control agent will have to be accompanied by a certificate of biological purity and a certificate of origin provided by the National Plant Protection Organization of the exporting country (Mason et al. 2005).

4. Present state, use, and perspectives of the genus *Fraxinus* in Mexico⁶

The genus *Fraxinus* is found in the main mountain areas of Mexico and, like many other Holarctic genera, reaches its southern limit in Central America. The Comisión Nacional para el Estudio de la Biodiversidad reports 21 species of *Fraxinus*, but a detailed assessment of their status still needs to be done. If synonymous names are removed, there are probably 13 species and one variety of *Fraxinus* in Mexico: *F. anomalla*, *F. berlandieriana*, *F. cuspidata*, *F. dipetala*, *F. dubia*, *F. gooddingii*, *F. gregii*, *F. papillosa*, *F. pringlei*, *F. purpusii* (var. *purpusii* and var. *vellerea*), *F. rufescens*, *F. udhei*, and *F. velutina*. The presence of two of them (*F. anomalla* and *F. dipetala*) in Mexico still needs to be confirmed, and the status of *F. pringlei* is doubtful and needs revision.

⁶ Abstract taken with the author's permission from: Bonfil, C. 2010. Present state, use, and perspectives of the genus *Fraxinus* in Mexico in: 2010 Proceedings Symposium on Ash in North America.

Many of the species found in northern Mexico are shared with the southwestern United States (*F. anomalla*, *F. berlanderiana*, *F. dipetala*, *F. gooddingii*, *F. gregii*, *F. papillosa*, and *F. velutina*). Three other species from central/south Mexico are also in Guatemala or even Honduras (*F. dubia*, *F. purpusii*, and *F. uhdei*). Only *F. rufescens* (and perhaps *F. pringlei*) is endemic to Mexico, where it grows in the central states.

Most species of *Fraxinus* are found in protected humid slopes in temperate (*Quercus-Pinus*) forests and as part of riparian vegetation, but species such as *F. uhdei* are also elements of moist forests. Still others (such as *F. rufescens*) are found in the transition between temperate and tropical deciduous forests and even in xeric shrub lands. *F. uhdei* is the only species that has a widespread distribution in Mexico, and therefore it is the only one that has been relatively well studied. It is a common urban tree, very abundant in Mexico City and Guadalajara, where it shows a weedy behavior. Because of its fast growth and high survival rate, it is a commonly cultivated species for reforestation programs. Ecological and forestry studies of other species present in Mexico still need to be done.

The main pest of *Fraxinus* in Mexico is *Hylesinus aztecus* Wood (Coleoptera, Curculionidae), a bark beetle, which has caused large declines in urban ashes. The emerald ash borer (*Agrilus planipennis*) has not been reported in Mexico, but its rapid spread through the United States makes it very probable that it will be able to reach Mexico in the near future.

5. How to Stimulate Research for the Application of Biological Control

Based upon the previous chapters, it would appear the major gaps in research knowledge are:

1. Understanding establishment, spread, and impact upon EAB of the Asian biological control agents in use in the US (note: this project is underway at some sites in the US)
2. A more thorough understanding of native parasitoids, pathogens, and predators
3. Specific guidance for seasonal timing and magnitude of releases to increase the efficacy of control actions
4. Understanding entomopathogen fungi, their ecological importance and role, sub-lethal dose and winter mortality
5. Develop entomopathogen fungi multiple uses (spray, auto-contamination and auto-dissemination)
6. Determine if natural abundance of entomopathogens (around 2% mortality) can be increased in natural sites
7. Document if identified fungus isolates share the same ecological zones
8. Type (positive, negative, or neutral) and magnitude of impact of entomopathogen fungi on native and introduced parasitoids
9. Trojan insects: Use of arthropods (ants, parasitoids, Acari) as vectors of entomopathogen to EAB larvae.

Additionally, research on the compatibility of various strategies, including biological controls, as part of an IPM program may be warranted. The US has undertaken a Slow Ash Mortality (SLAM) program, and the results of this work might be replicated in other countries and data pooled to provide long-term guidance on mitigation of EAB.

Finally, other management tools could be sought to complement the SLAM program. These may include:

1. Volunteer services in school program: ex: student project on rearing insect parasitoid in Biology class – for a summer release. Objective is education of younger people.
2. Increase the bird predation (mainly woodpeckers) with a nesting box building promotion in cities.
3. Evaluate the feasibility of sterile insect technique and mass-rearing of male EAB.
4. Improving pheromones lures & traps, study mating disruption of EAB;
5. Evaluate pheromones for use in monitoring establishment and efficacy of native and introduced biological control agents (parasitoids)

6. Management of EAB through Harmonization of Biological Control Efforts

6.1 Exchange of biological control experience

EAB biological control agents (including entomopathogens) should be shared amongst member countries wherever possible – small colony scions (or isolates) can be transferred from the US Rearing Facility, with US-PPQ + CFIA + PMRA approval, once appropriate permit processes are in place and the recipient country has a facility available for rearing. NAPPO could assist this effort by encouraging program officials in each member country to facilitate the transfer of insect (and entomopathogen) material, and support the use of biological control agents against EAB infestations.

Exchange could also be used for:

- Leveraging international collaborations to perform SLAM in different sites (areas) under common protocol and share data and results, including collaboration between USA, Canada and Mexico via CFIA, APHIS, CFS, USDA, provinces and states.
- Sharing entomopathogen material (cultures, strain information) between USA, Canada and Mexico
- Sharing information via NAPPO web site
- Harmonizing PMRA rules with APHIS rules. Agreements can be developed around products used in USA and Canada and Mexico.
- Discussions with specific Agencies involved in plant protection to share biological control tools.

6.2 Operational adjustments

As a partial alternative to Section 1, the US Rearing Facility, with US-PPQ approval, could supply limited quantities of parasitoids for introductions in other member countries, given all appropriate permits are in place. NAPPO could play a critical role by encouraging its member countries to make any operational changes required to facilitate this exchange.

6.3 Centralized repository of strategic knowledge

NAPPO could serve as a centralized repository of strategic knowledge (e.g. biological control program successes/failures) from which member country officials can draw, to optimize strategies when and where EAB is to be managed through biological controls. This site could reach the general public (as well as cities and foresters or conservation associations). Ex: How to survey for EAB (best practices), how to proceed when infestation is discovered, and material disposal solutions (including valorization).

7. Conclusions and Recommendations

According to the biology and current distribution of the EAB, eradication is not an option and our only alternative is to use Integrated Pest Management tools to slow the spread of this insect.

In this context:

1. Biological control for EAB is a promising strategy that has minimal environmental costs compared to traditional, chemical management strategies, and can be used in large natural areas where other management strategies are less feasible or economically impossible to implement.
2. EAB biological control agents (including entomopathogens) have shown ability to overwinter and establish in many areas of the US where EAB is present. This bodes well for areas of Canada, for example, that are broadly similar in climate to the northern US.
3. EAB biological Control should be adopted, wherever fiscally and operationally feasible, by member countries when and where EAB is detected.
4. Biological control must be considered as a long term strategy and we could hope that a natural equilibrium may be reached in several to many years, similar to that already observed in other entomological systems where exotic invasive insects are now “kept at bay” with diseases and parasitoids (ex: NPV and gypsy moth in eastern Canada, virus and European spruce sawfly, parasitoid and mountain ash sawfly.)

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