# Report 2010–12

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## Summary

1. Following a reassessment of the potential for biological control of *Rhamnus cathartica*, work in 2010–12 focussed on assessing the feasibility of using the psyllid *Trichochermes walkeri* and the seed-feeding midge *Wachtiella krumbholzi* as biological control agents, and determining the causes of the high levels of seedling mortality and post-dispersal seed mortality of *R. cathartica* observed in Europe as compared to North America.

2. In Europe, *R. cathartica* trees were found to be infected with '*Candidatus* Phytoplasma rhamni' ('*Ca.* Phytoplasma rhamni') at almost all surveyed localities, confirming previous reports of host association of this phytoplasma with *R. cathartica,* although the presence of witches' broom symptoms were not observed. Phytoplasma was not detected in any of the other *Rhamnus* species analysed, which suggests a very specific host association of this phytoplasma with its plant host, and also a very specific relationship between the insect vector of the pathogen and its host plant.

3. Work on '*Ca.* Phytoplasma rhamni' in North America has been carried out by Dr Roger Becker and Dr Dimitre Mollov, University of Minnesota, St Paul, USA. '*Ca.* Phytoplasma rhamni' has not been detected in 75 *R. cathartica* populations from North America suggesting either that the phytoplasma has not been introduced in the exotic range of its host plant, or that the absence of a suitable vector for phytoplasma propagation constrained its establishment in North America.

4. The absence of symptoms on all phytoplasma-infected trees could be an indication of a commensal relationship between the phytoplasma and its plant host, i.e. the absence of negative effects which would lead to the development of a disease in the host plant. Plants with asymptomatic presence of phytoplasma are considered to be a wild reservoir of the pathogen, since they are not affected by its presence.

5. *Trichochermes walkeri* proved to be infected with '*Ca.* Phytoplasma rhamni' at a very high rate in almost all sampled localities. Transmission trials strongly suggest that *T. walkeri* is not a vector of '*Ca.* Phytoplasma rhamni'. *Trichochermes walkeri* acquires the phytoplasma during feeding on infected plants, but it is not capable to re-inject the phytoplasma during feeding. The presence of phytoplasma in *Cacopsylla rhamnicola* and *Trioza rhamni* adults is reinforcing the need for elucidating the potential role of these psyllids in phytoplasma infection of *R*. *cathartica*.

6. To date, we have recorded the seed-feeding midge *W. krumbholzi* at most *R. cathartica* sites where we have looked for its presence; we found it at ten sites in Serbia, six sites in Austria, three sites in western Switzerland and two sites in southern Germany. Midge larvae have also been discovered in the fruits of *R*. *saxatilis* ssp. *tinctorius* at one site in Serbia, where *R. cathartica* also occurs. Based on the mitochondrial COI (cytochrome c oxidase) gene, midges from *R. cathartica* and *R. saxatilis* ssp. *tinctorius* are clearly two closely related but distinct species. This further confirms the likely high degree of host specificity of *W. krumbholzi.* Since we have not succeeded so far in obtaining even reproducing trees of the host, *R.*

*cathartica,* when grown in pots, we are doubtful whether it will be feasible to successfully screen *W. krumbholzi* in the near future.

7. An impact study of the effect of leaf galling by *T. walkeri* on eight-month-old *R. cathartica* seedlings was set up in August 2011. A total of 714 eggs were laid on infected trees. However, in 2012, no galls were recorded and the test was terminated without having obtained conclusive results.

8. We did not find evidence of negative plant–soil feedback by mature *R. cathartica* on conspecifics that could explain low seedling numbers of *R. cathartica* in the native range. There was however a positive plant–soil interaction in the rate of seedling emergence. A small difference in the number of days to seedling emergence probably explains most of the variation in seedling growth.

9. Due to the difficulties surrounding currently studied agents and the low probability of finding additional potential agents, it has been decided that the project will be stopped. A publication summarizing main results will be prepared until 2013.

# 1. Introduction

*Rhamnus cathartica* L. (common buckthorn) is a shrub or small tree native to much of Europe and western Asia that has successfully invaded many habitats in North America including abandoned agricultural fields, hedgerows, forest, field and wetland edges, and occasionally contiguous forest habitats (Kurylo *et al.*, 2007).

*Rhamnus cathartica* was introduced to North America as an ornamental shrub in the early 1800s and was originally used for hedges, farm shelter-belts and wildlife habitats (Gourley, 1985; Randall and Marnelli, 1996; Gale, 2001). It is now naturalized throughout the upper mid-western and north-eastern USA and the maritime provinces of Canada.

*Rhamnus cathartica* is bird-dispersed and dioecious (Godwin, 1943). It has a wide habitat tolerance but grows most quickly in areas with more light if moisture is not limiting (Knight *et al.*, 2007). The positive association between availability of light and seedling density in North American forest habitats also shows the importance of canopy openings in colonization by *R. cathartica* (McCay *et al.*, 2009).

Fruit production by *R. cathartica* in North America has been described as “very prolific” and “aggressive” (Knight *et al.*, 2007). As expected from the prolific fruit production and high germination rates of *R. cathartica*, high densities of seedlings may be found near parent shrubs in invaded areas (see (Knight *et al.*, 2007). While average number of seedlings was greater than 100/$m^2$ beneath a dense *R. cathartica* stand in Saskatchewan, Canada, we have only observed very low seedling density in Europe. A study in a plantation in England, where all mature *R. cathartica* shrubs were known to be reproducing yearly, found only 6.2 seedlings/$m^2$ under conspecific shrubs (Kollmann and Grubb, 1999).

Research to develop biological control for buckthorns was initiated in 1964 and preliminary screening tests were conducted in 1966–67 (Malicky *et al.*, 1970). A new programme was started in 2001 and has taken into consideration increasing concerns over potential non-target impacts of biological control agents and greater demands for high levels of specificity (Louda *et al.*, 1997; Pemberton, 2000).

Over 30 specialized arthropod species have been recorded on *R. cathartica* in Europe, including 21 Lepidoptera, six Hemiptera, two Diptera, one Coleoptera and three Acari. Less is known about fungal pathogens associated with this species. Based on a literature search and evaluation of herbarium records, a couple of potentially specific fungal pathogens are recorded on *R. cathartica,* which may too cause considerable damage to their host plant in the native range (Gassmann *et al*., 2001).

A literature review has indicated that Lepidoptera have been one of the least successful taxonomic groups for the biological control of shrubs and trees (Gassmann *et al*., 2010). In addition, the seven Lepidoptera we have investigated so far are either not sufficiently specific or very difficult to test (Gassmann *et al.*, 2008). Also, the only specialized beetle known on

buckthorn in Europe, the stem-boring longhorn beetle, *Oberea pedemontana* (Coleoptera: Cerambycidae), is not specific at the genus level.

Based on the results to date, the next best group to consider is the sap suckers. Nine species have been recorded on *R. cathartica* in Europe including three psyllids – *Trichochermes walkeri* (Hemiptera: Triozidae), *Cacopsylla rhamnicola* (Hemiptera: Psyllidae) and *Trioza rhamni* (Triozidae) – one Miridae (Hemiptera) and three Eriophydidae (Acari). With the exception of one species inducing leaf erinea on *R. cathartica*, none of the eriophyid mites has been observed on buckthorn in past surveys. The leaf-margin gall psyllid *Trichochermes walkeri* is currently being evaluated.

The detection of '*Candidatus* Phytoplasma rhamni' ('*Ca.* Phytoplasma rhamni') in *T. walkeri* adults in 2009 raises several questions that need to be addressed before further considering sap suckers for biological control of *R. cathartica*: (i) is the phytoplasma '*Ca.* Phytoplasma rhamni' common on *R. cathartica* in Europe, (ii) does '*Ca.* Phytoplasma rhamni' already occur in North America, and if yes, what is the vector, (iii) does the phytoplasma occur on other *Rhamnus* species in Europe, (iv) does *T. walkeri* transmit the phytoplasma, and if not, what is the vector, and (v) is '*Ca.* Phytoplasma rhamni' specific to *R. cathartica* as is suggested in the literature?

Another important group of potential agents, given the high seed output of *R. cathartica* in North America, are the seed feeders. Two midge (Diptera: Cecidomyiidae) species and two Lepidoptera are known to attack the fruits of *R. cathartica* in Europe (Gassmann *et al.*, 2001). One midge, *Wachtiella krumbholzi*, is under evaluation. We have not found the second midge species, *Lasioptera kosarzewskella*, or the two lepidopteran species, *Sorhagenia rhamniella* (Cosmopterigidae) and *Hysterosia sodaliana* (Tortricidae), which in addition do not appear to be genus specific according to the literature. *Wachtiella krumbholzi* is therefore the only available potential seed feeder for biological control of *R. cathartica* but the feasibility of host-range testing still needs to be addressed.

Following recommendations from an external group of experts, the project focussed in 2010–12 on (i) continuing to assess the feasibility of using the psyllids, in particular *T. walkeri*, and the seed-feeding midge *W. krumbholzi* as biological control agents (this includes additional studies of the phytoplasma '*Ca.* Phytoplasma rhamni'), and (ii) determining the causes of the high levels of seedling mortality and post-dispersal seed mortality of *R. cathartica* observed in Europe as compared to North America as a step towards identifying additional potential biological control agents including pathogens.

Work on '*Ca.* Phytoplasma rhamni' in North America has been carried out by Dr Roger Becker and Dr Dimitre Mollov, University of Minnesota, St. Paul, USA.

## 2. Phytoplasma '*Candidatus* Phytoplasma rhamni'

### 2.1. Introduction

Plant-pathogenic phytoplasmas are non-culturable, insect-transmitted, wall-less prokaryotes of the class *Mollicutes* that are associated with diseases in

several hundred plant species, including many woody shrubs or small trees (Marcone *et al*., 2004; Weintraub and Beanland, 2006). Based on conventional and computer-simulated RFLP (restriction fragment length polymorphism) analyses of 16S rRNA (ribosomal RNA) gene sequences, all phytoplasmas identified to date are classified within 30 main groups (designated 16SrI to 16SrXXX) and over 100 subgroups which are designated with a letter suffix (Zhao *et al*., 2010).

A lethal witches' broom disease of *R. cathartica* was observed for the first time in the Rhine Valley in south-western Germany in the 1990s (Mäurer and Seemüller, 1996). This disease, characteristic symptoms of which we have never observed, is caused by buckthorn witches' broom (BWB) phytoplasma, which belongs to the 16SrXX – BWB phytoplasma group (Wei *et al*., 2007), subgroup -A. BWB phytoplasma was previously classified within the 16SrX – apple proliferation group (AP) as subgroup -E (Lee *et al.*, 1998), due to the closer phylogenetic relatedness of BWB to the phytoplasmas of this group than to other phytoplasma subclades (see Marcone *et al.,* 2004 for references). Following a recently updated classification scheme, the 16SrX group of phytoplasmas currently includes the AP (16SrX-A), pear decline (16SrX-C), Spartium witches' broom (16SrX-D) and European stone fruit yellows (16SrX-F) phytoplasmas. On the other hand, the BWB phytoplasma is, on the basis of low RFLP pattern similarity with all known phytoplasmas in group 16SrX and other groups, assigned to the 16SrXX group (Wei *et al.*, 2007).

To resolve the taxonomic position of the phytoplasmas, a provisional taxonomic system for uncultured bacteria (Murray and Schleifer, 1994) was recently adopted for naming phytoplasma species candidates, within a genus-level taxon '*Candidatus* Phytoplasma' ('*Ca*. Phytoplasma') (IRPCM, 2004). So far, 32 '*Ca.* Phytoplasma' species have been formally described (Zhao *et al*., 2010 and references therein; Lee *et al.*, 2011; Malembic-Maher *et al.*, 2011; Davis *et al.*, 2012; Martini *et al.*, 2012). For uncultured phytoplasmas, a novel putative species may be described when its 16S rRNA gene sequence (>1200 base pairs) has ≤ 97.5% similarity to any previously described '*Ca.* Phytoplasma' species (IRPCM, 2004). The BWB phytoplasma shares < 97.5% 16S rDNA sequence similarity with other known phytoplasmas, including the AP group phytoplasmas. Thus Marcone *et al*. (2004) proposed the BWB phytoplasma as a novel '*Ca.* Phytoplasma' species, i.e. '*Ca.* Phytoplasma rhamni'. According to these authors, the BWB phytoplasma has clearly distinct molecular and biological properties, and in particular a different and unique field host plant, *R. cathartica*.

The single most successful group of insect vectors of phytoplasmas are the Hemiptera. Phytoplasmas are phloem-limited; therefore, only phloem-feeding insects can potentially acquire and transmit these pathogens. However, within the phloem-feeding Hemiptera only a small number, primarily in a very few taxonomic groups, have been confirmed as vectors of phytoplasmas (Weintraub and Beanland, 2006). The main group of known vectors is the Cicadellidae, although 15 species in another seven families are also known to be vectors of phytoplasmas (Weintraub and Beanland, 2006).

The importance of psyllids as possible vectors of phytoplasma diseases has been recognized only recently and comprehensive research on their role as vectors has been carried out in the past few years (reviewed in Jarausch and Jarausch, 2010). All confirmed and recognized psyllid vectors to date belong to a single genus, *Cacopsylla*. Five species of *Cacopsylla* are confirmed vectors and transmit AP group (16SrX) phytoplasmas on apple, stonefruit and pear trees: *C. picta*, *C. melanoneura*, *C. pruni*, *C. pyri* and *C. pyricola* (Jarausch and Jarausch, 2010). Another three species within this genus are considered as possible vectors: *C. pyrisuga*, *C. qianli* and *C. chinensis*; these latter *Cacopsylla* species were found to be infected with AP group phytoplasmas, but their vector role and transmission efficacy has yet to be clarified. Additionally, there are two reports of psyllid vectors belonging to genera other than *Cacopsylla,* both transmitting a phytoplasma to carrots. *Bactericera trigonica* was found to transmit a stolbur (16SrXII) phytoplasma and *Trioza nigricornis* to transmit the aster yellows (16SrI) phytoplasma. Nonetheless, since the vector role of these psyllids in phytoplasma transmission is not confirmed, at present they are treated only as tentative vectors.

In 2009 the presence of the phytoplasma '*Ca.* Phytoplasma rhamni' (16SrXX-A subgroup) was detected in *Trichochermes walkeri* adults from two localities in western Switzerland. Our goal in 2010–12 was (1) to sample *R. cathartica*, other *Rhamnus* species, *Frangula alnus* and the psyllids *T. walkeri*, *C. rhamnicola* and *Trioza rhamni* from a number of sites in western and south-eastern Europe and checkthem for the presence of phytoplasma, and (2) to carry out transmission trials with *T. walkeri*.

## 2.2. Materials and methods

### *2.2.1. Plant and insect collections*

Characteristic symptoms of witches' broom, which would indicate the presence of the phytoplasma, were not observed at any of the surveyed buckthorn sites. At some localities discrete leaf yellowing and/or small leaves were present on a few trees and these were sampled individually and treated as possibly symptomatic. All other sampled *R. cathartica* trees were asymptomatic.

When possible, five trees (samples) were sampled at each buckthorn site and 15–20 leaves collected per tree. One constraint was that plant material collected for the detection of phytoplasma should be neither dry nor mouldy. Leaf tissue was cut approximately 3 mm either side of the mid vein. For each sample, mid veins and petioles were put together in a plastic vial (8 cm long; 1.5 cm diameter). A small hole was made in the lid for ventilation. Collections were always sent within 24 hours to the Institute for Plant Protection and Environment in Belgrade, Serbia, for processing. Delays were encountered with some of the shipments, but the plant material was still in good condition even 8–10 days after collection when stored as described above.

For *R. cathartica*, five leaves from each tree sampled for phytoplasma detection were also sampled for molecular identification of plant genotype. For this purpose the leaves were placed in silica gel.

In July–August 2010, all buckthorn sites were carefully inspected for *T. walkeri* galls. Enough galls were collected at each site to give about 20 L4–L5 *T. walkeri* nymphs. Development of *T. walkeri* was delayed by at least ten days compared to previous years, and in Austria only L2–L4 could be collected. All samples were stored in 95% ethanol before being processed.

In June 2011 leaf samples of *R. cathartica* with galls of *T. walkeri* were collected at Šušara in Serbia and tested for phytoplasma presence. Phytoplasma-positive trees were selected for the collection of 100 *T. walkeri* adults in August 2011, with the assumption that these adults had harboured the phytoplasma. These specimens were used for transmission trials (see section 2.5), and were all subsequently analysed for phytoplasma presence.

In addition a few *C. rhamnicola* and *Trioza rhamni* adults were collected in 2010–11 at Griessheim, Germany and at Beranje, Serbia for phytoplasma detection.

### *2.2.2. Molecular detection and characterization of 'Ca.* Phytoplasma rhamni*'*

Total nucleic acids from plant midribs and petioles were extracted using a previously reported CTAB (cyltrimethylammonium bromide) protocol (Angelini *et al*., 2001). To identify phytoplasmas in *Trichochermes walkeri*, collected specimens were analysed in pools of 3–5 nymphs (depending on the stage) or individually in the case of adults. For *C. rhamnicola* and *Trioza rhamni* all collected specimens were adults and were analysed individually. DNA extraction from insects was performed using a modified CTAB method (Gatineau *et al*., 2001).

Phytoplasmas were detected in plant and insect DNA samples by polymerase chain reaction (PCR) amplification of the 16S rRNA gene using the universal phytoplasma and group specific primer pairs. Amplification was performed in nested PCR with P1/P7 primers (Deng and Hiruki, 1991; Smart *et al.*, 1996) followed by an F2n/R2 universal primer pair (Gundersen and Lee, 1996) or R16(X)F1/R1 primers specific for amplification of 16SrX group and related phytoplasmas (Lee *et al*., 1995). Amplicons obtained with F2nR2 primers were subjected to RFLP analyses with *Mse*I, *Alu*I and *Hpa*II endonucleases, following the previously described procedure of Lee *et al*. (1998). '*Candidatus* Phytoplasma rhamni' DNA isolated from naturally infected *R. cathartica* from a location between Neuhofen and Ludwigshafen in Rheinland-Palatinate, Germany (type locality of '*Ca.* Phytoplasma rhamni'; provided by Bernd Schneider, Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany) was used as a reference positive control in all reactions.

Characterization of detected phytoplasmas was performed by sequence analysis of the 16S rRNA gene and ribosomal protein gene operon consisting of the *rpl*22 and *rps*3 genes encoding ribosomal proteins L22 and S3. For sequence analysis of the 16S rRNA gene, amplification was conducted using P1/P7 primers in direct PCR, followed by nested PCR with the P1A/P7A primer pair, with reaction conditions according to Lee *et al*. (2004). Amplification and sequence analysis of ribosomal protein genes *l*22 and *s*3 was performed as described by Martini *et al*. (2007), with rpL2F3/rp(I)R1A primers used for direct PCR and followed by nested PCR with the rpF1C/rp(I)R1A primer pair.

## 2.3. Results and discussion

### *2.3.1. Plants*

'*Candidatus* Phytoplasma rhamni' was detected in 25% of all *R. cathartica* samples, at several sites in all countries surveyed, except for Montenegro, but not in any of the other three *Rhamnus* species sampled or in *F. alnus* (Jović *et al*., 2011) (Table 1; Annex 1).

**Table 1.** Geographic origin and number of *Rhamnus* spp. and *Frangula alnus* samples analysed with PCR results on '*Candidatus* Phytoplasma rhamni' presence.

| **Country** | **Number of plant samples positive/analysed** | | | | |
|---|---|---|---|---|---|
| | ***R. cathartica*** | ***R. alpina*** | ***R. saxatilis*** | ***R. rupestris*** | ***F. alnus*** |
| Switzerland | 14/35 | 0/20 | 0/0 | 0/0 | 0/19 |
| Germany | 3/25 | 0/0 | 0/0 | 0/0 | 0/0 |
| Austria | 11/30 | 0/0 | 0/3 | 0/0 | 0/0 |
| Serbia | 6/41 | 0/10 | 0/15 | 0/5 | 0/0 |
| Montenegro | 0/2 | 0/5 | 0/0 | 0/5 | 0/0 |
| Total | 34/133 | 0/35 | 0/18 | 0/10 | 0/19 |

We did not observe the witches' broom disease found previously on one occasion in Germany in the 1990s (Mäurer and Seemüller, 1996) and at present we cannot associate the presence of the phytoplasma with any particular symptoms. Our results have revealed a much wider geographic distribution of '*Ca.* Phytoplasma rhamni' than was previously known, which reinforced the need for a more detailed characterization of geographically distant isolates (Fig. 1). This is particularly important for elucidating '*Ca*. Phytoplasma rhamni' epidemiology, since it can be expected that different strains are transmitted by different vectors and with different rates of efficacy.

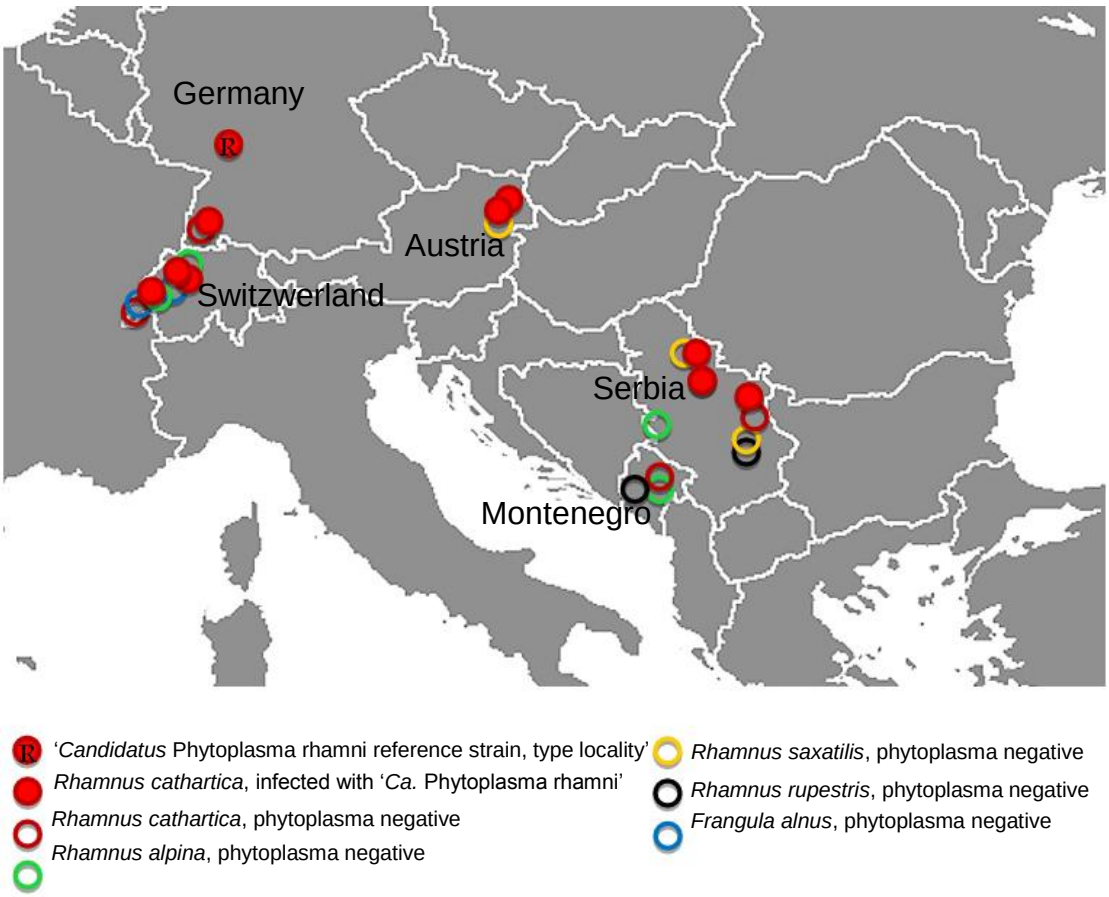


**Fig. 1.** Occurrence and geographical distribution of '*Candidatus* Phytoplasma rhamni' on *Rhamnus* spp. and *Frangula alnus* in Europe.

In order to trace the molecular variability of the phytoplasma and to identify possible strain differences we sequenced two phytoplasma gene fragments (16Sr RNA and *rpl22–rps3*) of selected '*Ca*. Phytoplasma rhamni' isolates which were geographically the most distant (Table 2). Comparison of 16Sr RNA gene sequences among isolates from Switzerland, Germany, Austria and Serbia showed that they share 100% identical nucleotide composition. Comparison with the reference BWB strain of '*Ca.* Phytoplasma rhamni' showed 99% identity, which was the consequence of the low quality of sequence read of this historical isolate which had several ambiguous nucleotide positions (Table 2, N positions). Phylogenetic analyses of '*Ca.* Phytoplasma rhamni' relatedness with closest relatives from AP group phytoplasmas confirmed the previously determined clear phylogenetic separation of these *Candidatus* species (pairwise distance ranged from 3.4% to 4.6%), which supports its biological uniqueness and specific host-plant association (Fig. 2).

**Table 2.** Nucleotide differences in 16S rRNA gene sequences in newly obtained and historical sequences of '*Candidatus* Phytoplasma rhamni' reference strain.

| Isolate | Locality | 16S rRNA nucleotide position [a] | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 553 | 573 | 944 | 945 | 1077 | 1104 | 1246 |
| BWB [b] | S Germany | N [c] | - | A | T | N [c] | N [c] | N [c] |
| 172-08-10 | SW Switzerland | C | C | - | - | C | A | G |
| 48-07-10 | NW Switzerland | C | C | - | - | C | A | G |
| 42-08-10 | SW Germany | C | C | - | - | C | A | G |
| 14-07-10 | NE Austria | C | C | - | - | C | A | G |
| 174-09-10 | NE Serbia | C | C | - | - | C | A | G |
| 207-09-10 | CE Serbia | C | C | - | - | C | A | G |
| 55-06-10 | E Serbia | C | C | - | - | C | A | G |

[a] Bases according to BWB (buckthorn witches' broom) reference strain (Marcone *et al*., 2004).
[b] Reference '*Ca.* Phytoplasma rhamni' strain BAWB (Marcone *et al*., 2004) GenBank Acc. number X76431 is highlighted in grey.
[c] N represents any nucleotide.

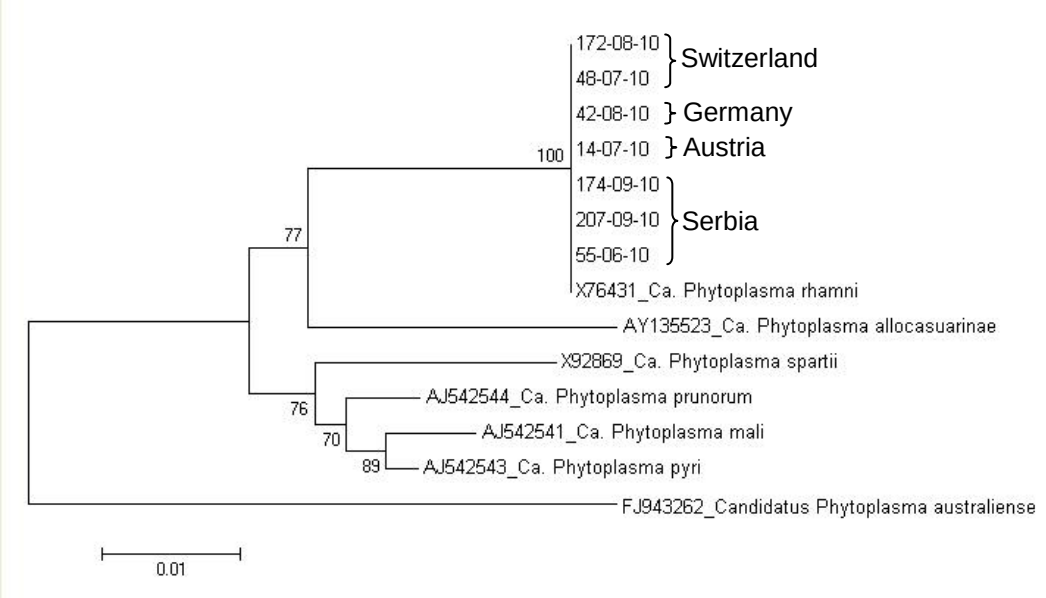


**Fig. 2.** Phylogenetic tree constructed by neighbour-joining method (p-distance model) inferred from 1358 base pairs of 16S rRNA gene fragments for seven '*Candidatu*s Phytoplasma rhamni' isolates from *Rhamnus cathartica* and reference strains of the 16SrX phytoplasma group ('*Ca.* Phytoplasma mali', '*Ca.* Phytoplasma pyri', '*Ca.* Phytoplasma prunorum', '*Ca*. Phytoplasma spartii', '*Ca*. Phytoplasma allocasuarinae'). '*Candidatus* Phytoplasma australiense' (16SrXII-B subgroup) was used as an outgroup to root the tree. Bootstrap values for 500 replicates are shown on branches. GenBank accession numbers of reference strains are indicated.

Sequence analyses of a ribosomal protein gene operon (*rpl*22–*rps*3 genes) of eight '*Ca*. Phytoplasma rhamni' isolates from different parts of western and south-eastern Europe (including the isolate from the species type locality in Germany) revealed a low level of intraspecific variability with only a single nucleotide change present in the sequence of isolates from north-west Switzerland and north-east Austria (Fig. 3). In contrast, interspecific

differences with reference sequences of 16SrX (AP group) phytoplasmas (‘*Ca.* Phytoplasma mali’, ‘*Ca.* Phytoplasma pyri’, ‘*Ca.* Phytoplasma prunorum’) confirmed the clear separation of ‘*Ca*. Phytoplasma rhamni’, with pairwise distances ranging from 16.3% to 16.6%. This high genetic divergence clearly confirms the independent evolution of ‘*Ca.* Phytoplasma rhamni’ from related phytoplasmas probably due to its specific adaptation to its environment (host plant). High genetic divergence also confirmed that ribosomal genes are genetic markers with higher resolution potential than the 16S rRNA gene which is the reason why they are useful in identification and separation of closely related strains. In the case of ‘*Ca*. Phytoplasma rhamni’, analysis of these marker genes revealed very low genetic variability.

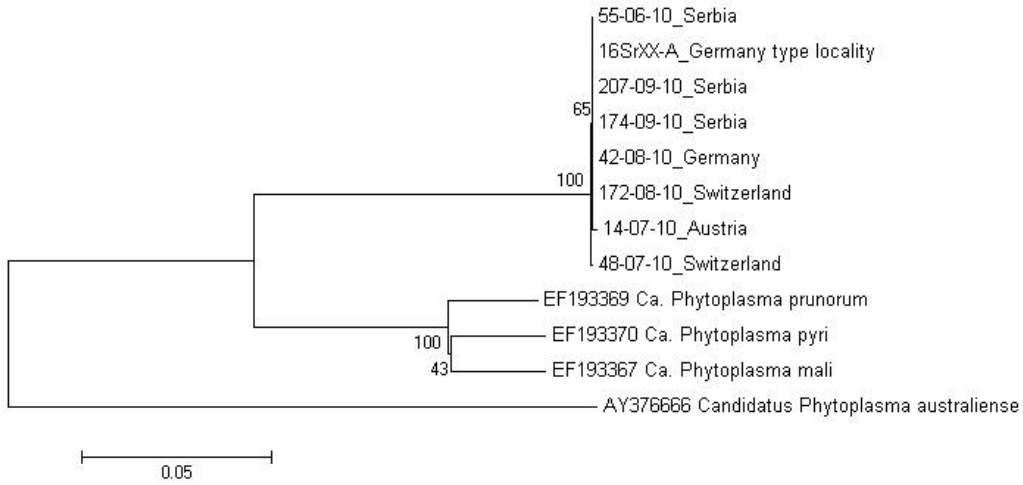


**Fig. 3.** Phylogenetic tree constructed by neighbour-joining method (p-distance model) inferred from 1048 base pairs of *rpl*22–*rps*3 genomic loci for eight ‘*Candidatus* Phytoplasma rhamni’ isolates from *Rhamnus cathartica* and reference strains of 16SrX phytoplasma group phytoplasmas (‘*Ca.* Phytoplasma mali’, ‘*Ca.* Phytoplasma pyri’, ‘*Ca.* Phytoplasma prunorum’). ‘*Candidatus* Phytoplasma australiense’ (16SrXII-B subgroup) was used as an outgroup to root the tree. Bootstrap values for 500 replicates are shown on branches. GenBank accession numbers of reference strains are indicated.

### *2.3.2. Insects*

Samples of *Trichochermes walkeri* from all collection sites in 2010 were positive for ‘*Ca.* Phytoplasma *rhamni’* except those from a very small site in Switzerland (CH10, see Annex 1) where neither *R. cathartica* nor *T. walkeri* tested positive. Interestingly, *T. walkeri* tested positive at site CH19, but the phytoplasma was not detected in any of the five heavily galled *R. cathartica* analysed. This is probably the consequence of an uneven distribution of phytoplasma bodies in the phloem of the infected trees, their low concentration (known for woody hosts in particular) and variations in titre according to the season and plant organ (reviewed in Firrao *et al.*, 2007), leading to false-negative results from the analysed plant samples.

From nine adult *C. rhamnicola* collected at a single site in Griessheim, Germany in 2010, one adult was found to be infected with ‘*Ca.* Phytoplasma

rhamni'. This is the first record of *C. rhamnicola* being infected with a phytoplasma (Table 3). In 2011, one adult *Trioza rhamni* from Serbia tested positive for '*Ca.* Phytoplasma *rhamni*'. Thus, all three psyllid species recorded from *R. cathartica* are tentative vectors of '*Ca.* Phytoplasma rhamni'.

**Table 3.** Detection of '*Candidatus* Phytoplasma rhamni' in three psyllid species recorded from *Rhamnus cathartica* in Europe.

| **Country** | **Date of collection** | **'*Candidatus*' phytoplasma rhamni' analysis (No. of positive pulls / No. tested)** | | |
|---|---|---|---|---|
| | | ***Trichochermes walkeri*** | ***Cacopsylla rhamnicola*** | ***Trioza rhamni*** |
| Austria | July 2010 | 34 / 36 | - | - |
| Switzerland | July–Aug 2010 | 84 / 98 | - | - |
| Germany | July 2010 | 10 / 10 | - | - |
| Germany | June 2010 | - | 1 / 9 | - |
| Germany | April 2011 | - | 0 / 2 | - |
| Serbia | July 2010 | 3 / 11 | - | - |
| Serbia | April 2011 | - | 0 / 3 | 1 / 7 |
| Serbia | August 2011 | 70 / 100 | - | - |

## 2.4. Transmission trials

*Trichochermes walkeri* proved to be infected with '*Ca.* Phytoplasma rhamni' at a very high rate in almost all localities sampled in 2010 (see Table 3). However, *T. walkeri* infection with phytoplasma only shows that this psyllid is acquiring the phytoplasma during feeding on infected plants, but not that it is capable of re-injecting the phytoplasma during feeding.

In 2011-2012, we carried out trials to test whether *T. walkeri* is capable of transmitting the phytoplasma to its host plant *R. cathartica.*

METHODS. Between 21 and 23 August 2011, a total of 100 *T. walkeri* adults were collected at Susara (South Banat, Serbia) from *R. cathartica* trees which had previously proven to be infected with '*Ca.* Phytoplasma rhamni'. Twenty *T. walkeri* adults were set up on each of three potted *R. cathartica* seedlings (eight-leaf stage). In addition 20 *T. walkeri* adults were set up on two other European *Rhamnus* species: *R. saxatilis* and *R. rupestris*. All adults were re-collected from the plants after 48 hours and subsequently preserved in 96% ethanol for detection of '*Ca.* Phytoplasma rhamni'. The exposed plants as well as two control plants of each of the species tested were kept in a mesh cage outdoors. All plants were analyzed by PCR for the presence of the phytoplasma on 7 April, 22 May and 3 September 2012.

RESULTS. Analyses of the *T. walkeri* adults used in the transmission trials confirmed the presence of phytoplasma in 70% of all specimens (Table 4). Twelve months after exposure to phytoplasma-infected *T. walkeri* adults, no symptoms could be observed on either of the *Rhamnus* tested. Control plants

were also asymptomatic. The repeated analysis in 2012 of all three *Rhamnus* species exposed to feeding by *T. walkeri* in 2011 did not reveal the presence of the phytoplasma. These results strongly suggest that *T. walkeri* is not a natural vector of '*Candidatus* Phytoplasma rhamni'.

**Table 4.** Results of transmission trials with phytoplasma infected *Trichochermes walkeri* adults on *Rhamnus* spp.

| | | ***Trichochermes walkeri*** | | **Phytoplasma detection in *Rhamnus* spp.** |
|---|---|---|---|---|
| | **No. of replicates** | **No. of adults released / replicate** | **Adult infection rate / replicate** | |
| *R. cathartica* | 3 | 20 | 65-70% | Negative |
| *R. saxatilis* | 1 | 20 | 75% | Negative |
| *R. rupestris* | 1 | 20 | 70% | Negative |

## 2.5. Detection of '*Ca.* Phytoplasma rhamni' in *Rhamnus cathartica* from North America (Roger Becker and Dimitre Mollov, University of Minnesota)

The potential use of *T. walkeri* as a biological control agent of *R. cathartica* in North America is complicated by the presence of '*Ca.* Phytoplasma rhamni'. It was therefore necessary to determine whether the phytoplasma already occurs in the introduced range of *R. cathartica*.

METHODS The leaf sampling protocol was similar to that used in Europe. In contrast to work done in Europe, a composite sample of several trees per site was used to detect the presence of the phytoplasma in buckthorn. Most samples were from Minnesota, with a few from Indiana, Michigan, Iowa and Wisconsin. All samples were processed using the Qiagen kit DNA extraction protocol and nested PCR (Lee *et al*., 1995; Smart *et al*., 1996). Two rounds of PCR reactions, (i) general phytoplasma primers and (ii) phytoplasma group X specific primers, were performed.

RESULTS None of the 75 buckthorn sites tested was found to have the buckthorn phytoplasma while a positive control sample obtained from Jelena Jović (Institute for Plant Protection and Environment, Serbia) was included each time and gave the expected size (positive) band. It can therefore be concluded that '*Ca.* Phytoplasma rhamni' does not occur in North America.

## 2.6. General discussion

*Rhamnus cathartica* trees were found to be infected with '*Ca.* Phytoplasma rhamni' at almost all surveyed localities, confirming previous reports of a host association between this phytoplasma and *R. cathartica*, although witches' broom symptoms were not observed. Phytoplasma was not detected in any of the other *Rhamnus* species analysed, nor in *F. alnus*, which could indicate a very specific host association between this phytoplasma and its host plant, as well as a very specific relationship between the insect vector of the pathogen and its host plant.

'*Ca.* Phytoplasma rhamni' has not been detected in *R. cathartica* populations from North America suggesting either that the phytoplasma has not been introduced in the exotic range of its host plant, or that the absence of a suitable vector for phytoplasma propagation constrained its establishment in North America.

The absence of symptoms on all phytoplasma-infected trees could be an indication of a commensal relationship between the phytoplasma and its plant host, i.e. the absence of negative effects which would lead to the development of a disease in a host plant. Plants with asymptomatic presence of phytoplasma are considered to be a wild reservoir of the pathogen, since they are not affected by its presence.

*Trichochermes walkeri* proved to be infected with '*Ca.* Phytoplasma rhamni' at very high rates in almost all sampled localities. Even young instars (L2–L3) collected in Austria were found to be infected with phytoplasma. Our transmission trials strongly suggest that *T. walkeri* is not a natural vector of the phytoplasma, and that the high infection rate detected in this species is the result of a very close host–plant association of this psyllid with *R. cathartica. Trichochermes walkeri* acquires the phytoplasma during feeding on infected plants, but it is not capable to re-inject the phytoplasma during feeding. No psyllid of the genus *Trichochermes* has previously been found to even harbor a phytoplasma, let alone transmit it. In addition, all confirmed psyllid vectors of phytoplasmas belong to the genus *Cacopsylla*, and in our field survey one *C. rhamnicola* adult was found to be infected with '*Ca.* Phytoplasma rhamni'. Although the limited number of specimens analysed does not yet allow us to draw conclusions, the presence of the phytoplasma in *C. rhamnicola* reinforces the need for elucidating its possible role as a vector of '*Ca.* Phytoplasma rhamni'.

Recently, '*Ca*. Phytoplasma rhamni' was also detected in *Cacopsylla myrthi* Puton during a survey for vectors of '*Ca*. Phytoplasma trifolii' (16SrVI group) on solanaceous crops in Lebanon (Choueiri *et al*., 2007). *Cacopsylla myrthi* was collected on *R. cathartica* (X. Foissac, pers. comm., 2011) and one of 13 analysed pulls of this psyllid was infected with '*Ca*. Phytoplasma rhamni'. This further suggests that species in the genus *Cacopsylla* could be the major vectors of '*Ca*. Phytoplasma rhamni' on *R. cathartica*. We could not find any record of *T. walkeri* in Lebanon.

Finally the phytoplasma has also been detected in *Trioza rhamni* thus showing that the three psyllid species associated with *R. cathartica* are able to acquire the phytoplasma during feeding. Previous to our research, only one species in the genus *Trioza*, *T. nigricornis,* had been found to harbour a phytoplasma, aster yellows phytoplasma (16SrI group), but the vector role of this species was not confirmed and it is treated only as a tentative vector. In a system where three psyllid species are probably associated with one unique host plant carrying a phytoplasma, it is unlikely that all three species would be the vector of the pathogen, at least not within the same epidemiological cycle. Given the known and well-documented vector ability of *Cacopsylla* spp. to transmit phytoplasmas from the AP group and the evolutionary relatedness of '*Ca*. Phytoplasma rhamni' with the AP group phytoplasmas, it can be

expected that feeding by *Cacopsylla* species on *R. cathartica* might play a major vector role in the transmission of the phytoplasma.

## 3. Impact of Leaf Galling by *Trichochermes walkeri* on the Growth of *Rhamnus cathartica* Seedlings

METHODS To determine the potential impact of *T. walkeri* on young buckthorn plants, ten eight-month-old *R. cathartica* were exposed to three field-collected pairs of *T. walkeri* on 17 August 2011. Another ten plants were used as controls. The number of eggs laid by the psyllids was recorded in late October 2011. The number of galls and the impact of leaf galling on plant growth were assessed in spring 2012.

RESULTS Two plants without eggs were discarded. The number of eggs recorded on each replicate is shown in Table 5. Previous oviposition experiments indicated that about 10% of eggs laid resulted in gall and larval development the following year, however no galls were recorded in 2012 and the impact experiment was terminated without having obtained conclusive results.

**Table 5.** Impact study with *Trichochermes walkeri* in 2011–12.

| Plant | Shoot height (cm) | No. of leaves | No. of eggs | No. of adults alive, 14 October 2011 | Gall and larval development in 2012 |
|---|---|---|---|---|---|
| **Test plants** | | | | | |
| No. 1 | 26 | 14 | 107 | 1 ♀ / 1 ♂ | 0 |
| No. 10 | 29 | 13 | 34 | 0 | 0 |
| No. 11 | 29 | 14 | 15 | 0 | 0 |
| No. 16 | 32 | 14 | 62 | 1 ♀ | 0 |
| No. 22 | 37 | 17 | 57 | 0 | 0 |
| No. 23 | 36 | 16 | 53 | 1 ♀ | 0 |
| No. 26 | 36 | 14 | 188 | 2 ♂ | 0 |
| No. 29 | 36 | 16 | 198 | 1 ♂ / 2 ♀ | 0 |
| Mean ± SD | 32.6 ± 4.2 | 14.8 ± 1.4 | | | |
| **Control plants** | | | | | |
| No. 2 | 34 | 11 | - | - | - |
| No. 7 | 29 | 14 | - | - | - |
| No. 9 | 32 | 14 | - | - | - |
| No. 13 | 28 | 12 | - | - | - |
| No. 17 | 35 | 15 | - | - | - |
| No. 21 | 38 | 14 | - | - | - |
| No. 24 | 28 | 12 | - | - | - |
| No. 25 | 33 | 12 | - | - | - |
| No. 28 | 40 | 19 | - | - | - |
| No. 30 | 41 | 16 | - | - | - |
| Mean | 33.8 ± 4.8 | 13.9 ± 2.4 | | | |

# 4. *Wachtiella krumbholzi* (Diptera: Cecidomyiidae)

## 4.1. Background

Little is known about this insect, which was identified by Dr M. Skuhrava (Czech Republic). Interestingly, with the exception of a few specimens reared from *R. cathartica* in the Czech Republic, Skuhrava has not found this species during 50 years of investigations on cecidomyiids in 1800 European localities (Simova-Tosic *et al*., 2000, 2004; Skuhrava *et al.*, 2005).

The main characteristics of fruits attacked by *W. krumbholzi* resemble premature fruit maturation in terms of changes in colour, but the fruits are larger in size and irregularly shaped. Attacked fruits become dark-red/black while healthy fruits remain green (Plate 1). Casual observations revealed up to nine midge larvae per fruit and three larvae in one seed. Once mature, the midge larva leaves the fruits and enters the soil to prepare a larval cocoon made of silk and debris.

**Plate 1.** Healthy fruits of *Rhamnus cathartica* (left) and attacked fruits (with mature larvae) (right); Griessheim, Germany, 20 July 2009.

Preliminary oviposition tests in 2009 indicated successful oviposition and larval development by *W*. *krumbholzi* in the very young developing fruits of *R. cathartica*. In contrast, no oviposition occurred in the one-month-older well-developed fruits.

## 4.2. Molecular characterization

To date, we have recorded *W. krumbholzi* in most *R. cathartica* sites where we have looked for it, i.e. we found it at ten sites in Serbia, six sites in Austria, three sites in western Switzerland and two sites in southern Germany. Midge larvae have also been discovered in the fruits of *R. saxatilis* ssp. *tinctorius* at one site in Serbia, where *R. cathartica* also occurs. Based on the mitonchondrial COI (cytochrome c oxidase) gene, midges from *R. cathartica* and *R. saxatilis* ssp. *tinctorius* are clearly two closely related but distinct species (Fig. 4). The mean genetic differences between the two species ranged between 2.7% and 3.7%. Strict host association with high genetic divergence between the two midge species has thus been confirmed at this sympatric site where both *Rhamnus* species co-occur.

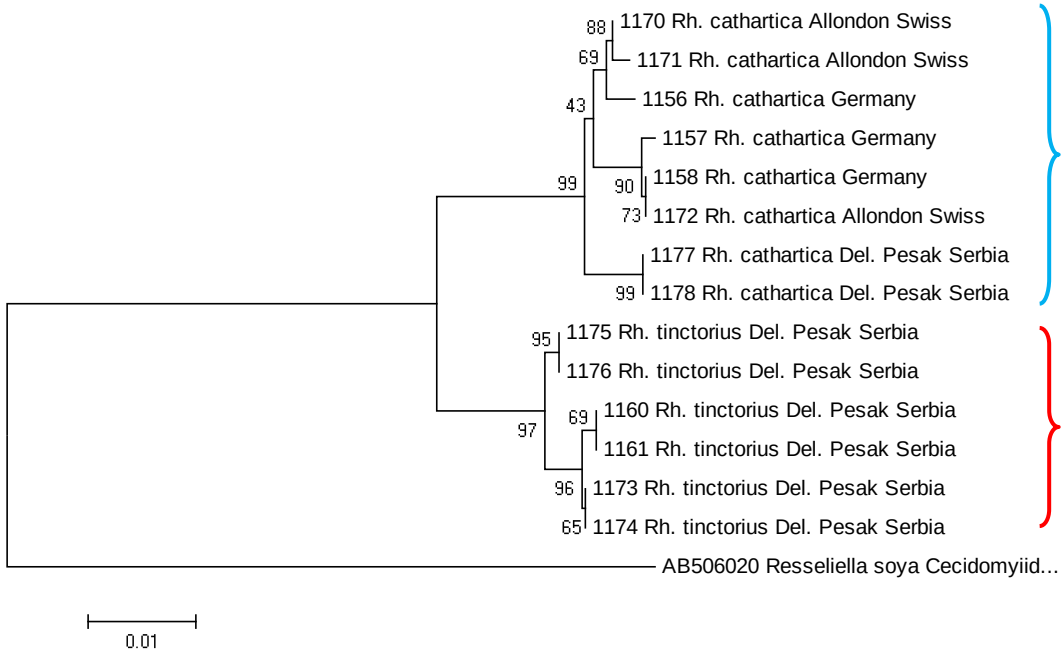


**Fig. 4.** Neighbour-joining phylogenetic tree (p-distance model) inferred from 626 base pairs of the mitochondrial cytochrome c oxidase gene for 14 midge specimens from *Rhamnus cathartica* and *R. saxatilis* ssp. *tinctorius*. The percentages of replicate trees in which taxa clustered together from a bootstrap analysis (500 replicates) are indicated (values lower than 40% are omitted).

No midge larvae were reared from the fruits of *F. alnus* collected in 2008 at one site in Austria and one site in Switzerland, where *R. cathartica* and *F. alnus* co-occur.

### 4.3. Adult emergence

In the past few years, emergence of gall midge adults in an outdoor shelter started about mid-May and was completed by early June. Adults started to emerge from gall midge cocoons, which had been stored at 3°C until late spring, two weeks after being moved to either the outdoor shelter or to controlled conditions of 20°C and emergence was completed within eight days. In 2009, adult emergence from larval cocoons stored at 1°C until early June started three weeks after they were returned to outdoor conditions and was completed within ten days. This indicates that we have the capacity to manipulate adult emergence to a certain extent. We also observed that cold treatment (10°C) was lethal for adults ready to emerge.

In 2011, 30 adult *W. krumbholzi* emerged in our outdoor shelter between mid-May and mid-June from about 200 fruits of *R. cathartica* collected in southern Germany in late summer 2010. Unfortunately, none of our potted *R. cathartica* flowered or fruited, thus no oviposition tests could be carried out.

### 4.4. Conclusions and outlook

Host-range tests with this fruit-attacking gall midge species relies entirely on oviposition tests. Synchronization between adult midge emergence and the availability of fruits in a very early phenological stage suitable for oviposition is therefore paramount. Our main difficulty is, however, to obtain fruits on potted *Rhamnus* species, which are mostly dioecious (i.e. male and female flowers are on separate plants). In addition, it takes several years for these trees/shrubs to reproduce. Since we have not so far succeeded in obtaining even reproducing trees of the host, *R. cathartica,* when grown in pots, we are doubtful whether it will be feasible to successfully screen *W. krumbholzi* in the near future.

## 5. Post-dispersal Seed and Seedling Mortality

### 5.1. Introduction

The interactions between plants and their soil community can result in dynamic feedbacks (Reinhart *et al*., 2003; McCarthy-Neumann and Kobe, 2010). Positive or negative soil feedbacks occur when a plant promotes a soil community that in turn benefits or inhibits conspecific plant performance compared with heterospecifics. Differences in interactions between native versus exotic plants and resident soil may play an important role in biological invasions and the persistence of exotic species (Nijjer *et al*., 2007). For example, the invasion of *Prunus serotina* in Europe is facilitated by the associated soil community (Reinhart *et al*., 2003). In the native range in the USA, the soil community that develops near *P. serotina* inhibits the establishment of neighbouring conspecifics and reduces seedling performance. This mechanism does not exist in the invaded range in Europe where the soil community enhances the growth of *P. serotina* seedlings.

The effect of mature *R. cathartica* shrubs on the growth and survival of seedlings is controversial (Knight *et al*., 2007). Some studies have shown a positive or neutral effect of mature conspecifics. Other studies have shown a negative effect presumably due to a strong shade effect of dense mature thickets. However, the potential accumulation of species-specific microbial communities in soil associated with the roots of adult trees has not been studied and the potential benefit of mycorrhizal colonization in the native versus invaded ranges of *R. cathartica* and the relative effects on *R. cathartica* and native plants remains unknown (Knight *et al*., 2007).

In 2011, we tested the hypothesis that seedling emergence and seedling performance of *R. cathartica* in Europe is affected by negative plant–soil feedbacks. We have conducted a greenhouse experiment on seedling emergence and seedling performance of *R. cathartica* using sterile versus non-sterile soils from two buckthorn sites in Switzerland.

### 5.2. Materials and methods

Approximately 1000 mature fruits of *R. cathartica* were collected at Griessheim in Germany on 3 September 2010 and at Cheyres and La Sauge in Switzerland on 9 September 2010. Fruit tissue was removed within three

days, seeds air-dried for 24 hours and stored in a paper bag at 4°C. A germination trial was set up on 19 November 2010 and 14 January 2011, 2.3 and 4.2 months after vernalization, respectively. Seeds from Griessheim germinated very poorly and were excluded from the experiment.

Soil samples were collected on 10 May 2011 at the two Swiss sites. At each site, a 5-litre soil sample was taken from the tree base of five mature female *R. cathartica* trees (the so-called tree effect) (Plate 2). A control soil sample was taken at a distance of 5 m from each sampled tree, making sure that no other *R. cathartica* tree was within 5 m of where the control sample was taken. All control samples were taken in the same habitat as the sample tree (i.e. forest or forest margin) with one exception, where the control sample was collected in the neighbouring wetland a couple of metres away from the forest edge. Vegetation cover was usually high around all trees and no seedlings could be seen within a distance of 5 m of any of the sampled trees. Each soil sample was air-dried for 24 hours, and then hand mixed and prepared by removing living macro-invertebrates, large organic particles and stones. In order to homogenize the samples, we sifted them through a 2-mm mesh sieve.

Each soil sample was air-dried for another 24 hours before being split into two equal parts. Soil to be sterilized was taken on 12 May to LEONI Studer Hard AG in Switzerland for gamma sterilization (max 50kGray, min 29kGray, Dosimeter Type Alanine 01/11). The non-sterilized soil was kept under the same conditions as the sterilized soil, i.e. in closed plastic boxes in the laboratory until being used in the germination trays. The experiment with non-sterile soil was set up on 24 May 2011 and with sterilized soil on 27 May, due to problems with the sterilization process.

Germination trays (48 × 25 × 6 cm) were separated into three parts by two pieces of wood (Plate 3). The two external parts (germination compartments) were used for the experiment. The middle part was left empty to avoid exchanges of particles between the germination compartments. The size of each germination compartment was about 15 × 25 × 6 cm containing approximately 2.2 litres of soil.

Seeds were surface-sterilized, the day before being sown, in a 7% sodium hypochlorite (Javel) solution for 3 min, and then rinsed with tap water for 30 sec before being soaked twice for 30 sec in sterilized (boiled) water (Chen, 2010). Forty seeds and 36 seeds respectively for soil from Cheyres and La Sauge were planted in four rows in each germination compartment (within the top 0.5 cm of soil) and covered with a fine layer of sand from a calcareous gravel pit obtained from a local commercial supplier.

The trials were set up as a full-factorial experiment with three factors: (i) site (Cheyres and La Sauge), (ii) *Rhamnus* presence (soil samples collected from underneath mature *Rhamnus* trees vs. control samples), and (iii) sterilization (sterilized vs. non-sterilized soil), resulting in eight treatment combinations. Each treatment combination was replicated five times, resulting in a total of 40 germination compartments. Each replicate was randomly assigned to each of the 40 germination compartments. Germination trays were gently watered as necessary and their position in the greenhouse changed randomly twice a week.

Seedling emergence was recorded 4–5 times every week and each seedling tagged using a toothpick (Plate 3). Between 12 and 15 September, seedling height was measured and the number of leaves (excluding cotyledons) counted. Seedlings were then harvested, dried for 24 hours at 80°C, and the aboveground and belowground biomass then measured immediately. The number of days to seedling emergence from the non-sterile soil was modified to take into account the three days' difference between set up of the experiment with sterile and non-sterile soils. Dead seedlings were excluded from the calculation of the mean dry weight.

**Plate 2.** M. Bennett, M. Penic and A. Leroux collecting soil samples within buckthorn stands at La Sauge, 10 May 2011.

**Plate 3.** Seedlings of *Rhamnus cathartica* in germination trays on 11 August 2011, 11 weeks after seeds were sown.

On 18 July, soil samples were collected in each germination compartment for mineral nitrogen ($NO_3^-$-N and $NH_4^+$-N) analysis. Each sample consisted of two cores of soil giving about 20 g of fresh weight. Cores were taken without disturbing seeds or seedlings and placed in plastic bags. Holes in the germination compartments were then refilled with the sand used to cover the

surface of the trays. On 19 July, the samples were taken to the Swiss Federal Institute of Technology in Zurich (ETHZ) to extract the mineral nitrogen. Analyses were carried out in the laboratory of the plant ecology group (Prof. P.J. Edwards) with the assistance of S. Güsewell and B. Jahn. About 10 g of soil was taken from each sample, weighed and placed in a glass jar together with 40 ml of calcium chloride extraction solution, closed with a lid and placed in a mechanical shaker for 60 min. The content of each jar was then filtered for nearly one hour and stored at 4°C. The total $NO_3^-$-N and $NH_4^+$-N content was measured on 20 July in a photometric analyser. Two additional blank jars were added as a control.

The experiment was analysed as a nested split-plot design using a linear mixed-effects model with site, soil and sterilization as fixed factors (see above) and replicate as a random factor. Differences between treatment combinations were assessed using Tukey's HSD. The relationship between the number of days for seedling emergence and the total biomass of the plant at the end of the experiment was analysed using linear regression. The nitrogen content and plant biomass data were $\log_{10}$-transformed prior to analysis. All analyses were done in R 2.13.0 (R Development Core Team, 2011).

## 5.3. Results and discussion

Non-sterile soil had a higher mineral nitrogen content than sterilized soil (19.9 ± 3.4 versus 11.8 ± 1.2 mg kg $soil^{-1}$), which was mainly due to a higher mineral nitrogen content at one of the sampling spots at Cheyres (Fig. 5; Annex 2). No correlation was found between the mineral nitrogen content and any of the plant parameters measured, thus the variation in the mineral nitrogen between sites or between sterile and non-sterile soils does not explain any of the results obtained.

On average, percentage of emerged seedlings was lower in sterile soil than in non-sterile soil (60.6 ± 1.2% versus 70.6 ± 2.1%) and higher at La Sauge than at Cheyres when soil was not sterilized (Fig. 5; Annex 2). This is probably because of the negative effect of sterilization on soil microbes that degrade the seed coat and thus facilitate seed germination and seedling emergence. Our data do not provide any evidence for pathogenic microorganisms causing pre-emergence seedling mortality, as shown for example with *Prunus serotina* in some North American sites (Reinhart *et al*., 2005).

Time to seedling emergence was not different in sterile and in non-sterile soils (52.1 ± 0.7 versus 54.6 ± 0.9 days) (Fig. 6; Annex 2). Times to seedling emergence or germination time are seldom considered in plant–soil feedback studies. Andonian *et al.* (2011) found no effect of soil sterilization on germination time of *Centaurea solstitialis* but a significant effect of soil regions. In contrast, de la Pena *et al.* (2010) did not find a significant effect of site or soil biota on the germination of the ground-hugging succulent perennial *Carpobrotus edulis*. In its exotic range in North America, seeds from *R. cathartica* trees growing in oak (*Quercus* spp.) woods germinated two weeks faster and had higher germination rates than seeds from neighbouring wetlands (Gourley 1985, in Knight *et al.*, 2007).

All plant parameters were greater in sterile soils than in non-sterile soils (Fig. 6; Annex 2). A negative correlation between time to seedling emergence and plant biomass was found (Fig. 7). This correlation suggests that plants in sterile soils were larger and had more leaves because they had slightly longer to grow than plants in non-sterile soils. It is likely that this apparent negative plant–soil feedback on seedling growth would be reduced should the seedlings be allowed to grow for a longer time before being harvested.

Finally no tree effect could be seen, indicating that the microbial communities were similar in buckthorn areas and buckthorn-free areas and the plant–soil interactions found at the two study sites are not the result of microorganisms associated with *R. cathartica*.

In summary, a positive plant–soil interaction was found in the rate of seedling emergence. The small, non-significant difference in time to seedling emergence probably explains most of the variation in seedling growth within the growing period under study.

Thus, we did not find evidence of negative plant–soil feedback of mature *R. cathartica* on conspecifics that could explain low seedling numbers of *R. cathartica* in its native range. However, this study suggests a balance of positive and negative interactions between *R. cathartica* and the soil biota that may contribute to give buckthorn a competitive advantage in a changing environment. Novel interactions between *R. cathartica* and resident soil organisms in the introduced range could generate benefits for the invader compared to the native plants.

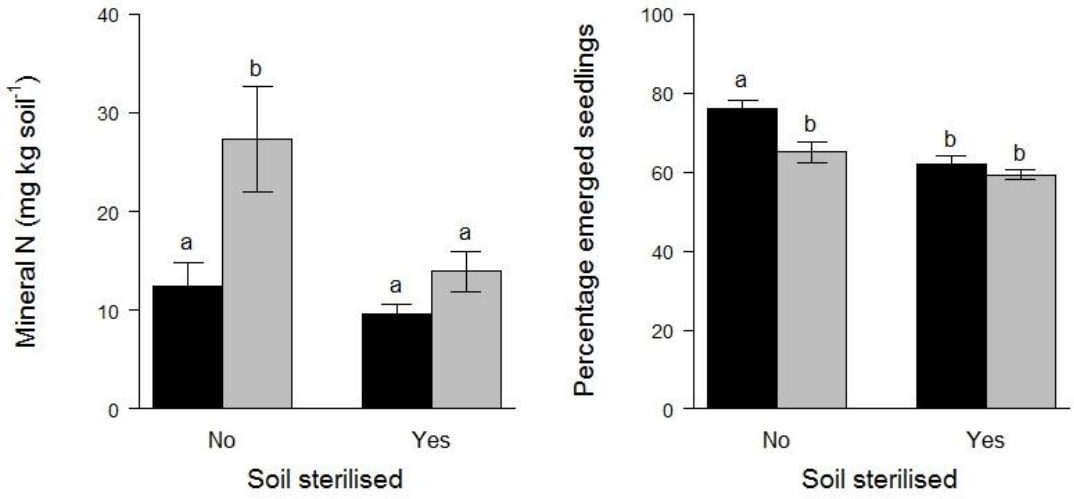


**Fig. 5.** Total mineral nitrogen content (left) and percentage of emerged seedlings of *Rhamnus cathartica* (right) in sterile and non-sterile soils from two Swiss sites in 2011. Grey and black bars indicate means for Cheyres and La Sauge, respectively. Error bars indicate one SE and small characters above the bars indicate significant differences (Tukey's HSD, $P < 0.05$).

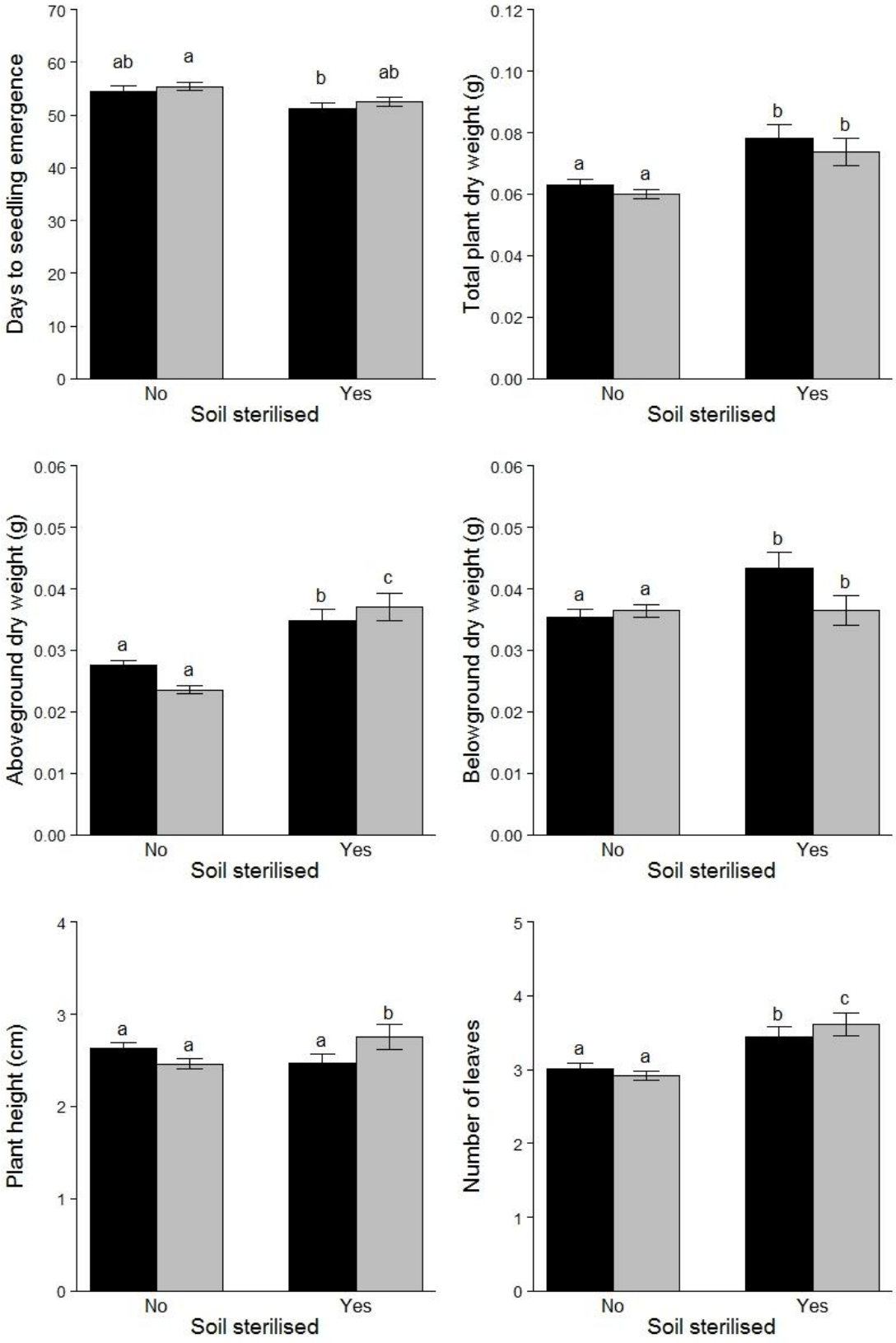


**Fig. 6.** Time to seedling emergence and seedling growth of *Rhamnus cathartica* in sterile and non-sterile soils from two Swiss sites in 2011. Shading as in Fig. 5. Error bars indicate one SE and small characters above the bars indicate significant differences (Tukey's HSD, $P < 0.05$).

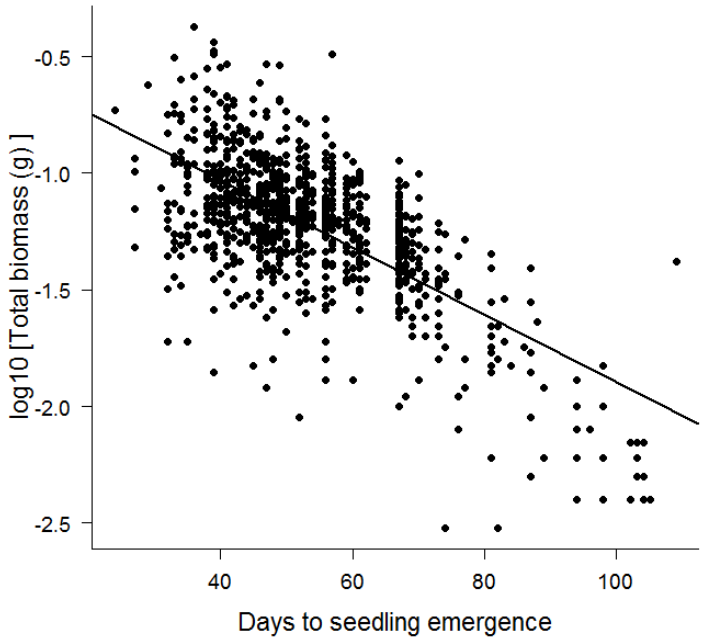


**Fig. 7.** Total dry weight of *Rhamnus cathartica* seedlings grown in soils from two Swiss sites in 2011 as a function of the number of days to seedling emergence ($R^2$ = 0.43, *n* = 916, *P* < 0.001).

## 6. Discussion

Although phytoplasma-infected trees have been identified over a wide geographic area within the natural range of *R. cathartica*, the phytoplasma has not been detected in any of the sampled buckthorn populations covering a wide geographical area from North America. One question is whether the phytoplasma has not been introduced into North America or whether the absence of a specific transmission mechanism prevented the pathogen to establish and spread in buckthorn populations in the introduced range.

Although all nymphal stages, as well as adults of *T. walkeri*, were found to harbour '*Ca.* Phytoplasma rhamni', this finding only indicates intensive feeding by *T. walkeri* on *R. cathartica* (which was infected at these locations) and their very close association. Transmission trials strongly suggest that *T. walkeri* is not a vector of '*Ca.* Phytoplasma rhamni'. No psyllids from the genus *Trichochermes* have ever been found to transmit any phytoplasma, while all currently recognized psyllid vectors belong to the genus *Cacopsylla*. The presence of '*Candidatus* Phytoplasma rhamni' in *Cacopsylla rhamnicola* and *Trioza rhamni* reinforces the need to elucidate the epidemiology of the phytoplasma, especially the vector role and transmission efficacy of these two psyllids as well as the host-plant specificity of '*Ca.* Phytoplasma rhamni' to *R. cathartica* and its congeners. Phylogenetic analyses of 16S rRNA and *rpl*22–*rps*3 genes of '*Ca*. Phytoplasma rhamni' and related phytoplasmas, further support its uniqueness and the clear separation of this phytoplasma from its

relatives, probably due to the specific transmission route and host-plant association. Given that this phytoplasma was described as causing a witches' broom disease in its host, its impact on *R. cathartica* and other *Rhamnus* species would need to be tested under controlled conditions. The absence of symptoms in transmission trials and in infected trees on all surveyed sites, suggests a commensal association between the phytoplasma and *R. cathartica*, with *R. cathartica* serving as a wild reservoir of the phytoplasma.

The occurrence of '*Ca.* Phytoplasma rhamni' in the three psyllids associated with *R. cathartica* makes the use of any of these potential agents more complicated. However, because our transmission trials indicate that *T. walkeri* does likely not transmit the phytoplasma, we believe that this probably very specific insect still has potential as an agent for *R. cathartica*.

Assessing the host specificity of *T. walkeri* relies on oviposition and larval development tests. Adult feeding and oviposition of *T*. *walkeri* are restricted to species in the genus *Rhamnus*. The likelihood of *T. walkeri* accepting a non-target species for oviposition in containment that would not be accepted in the field (a false positive) is considered high. *Trichochermes walkeri* has been recorded exclusively on *R. cathartica* in Europe. However, it must be noted that only a few *Rhamnus* species occur in its native range in Europe. Specific requirements for host acceptance and suitability will probably be related to stage of developing leaf bud, leaf shape and toughness as well as habitat. There are indications that larvae of *T. walkeri* will not complete development on small tough or thick evergreen leaves such as those of *R. alaternus*. Therefore, the native North American *Rhamnus* species *R. crocea*, *R. ilicifolia*, *R. serrata* and *R. smithii* are unlikely to be suitable for development of *T. walkeri* nymphs to the adult stage. Critical native non-target North American species for *T. walkeri* are *R. alnifolia* and *R. lanceolata* because of their leaf shapes and leaf smoothness, and their geographical distributions which partially overlap that of *R. cathartica.*

The challenges in working with the seed-feeding midge *Wachtiella krumbholzi* will be obtaining reproductive trees, pollination of female buckthorn flowers and synchronizing fruit development with midge oviposition and larval development. Since we have not so far succeeded in obtaining even reproducing trees of the host, *R. cathartica,* when grown in pots, we are doubtful whether it will be feasible to successfully screen W*. krumbholzi* in the near future. More generally, one current constraint in developing biological control of buckthorns is the difficulty of obtaining seeds for a number of test plant species and/or growing plants from seeds.

Finally, we did not find evidence of a negative plant–soil feedback of mature *R. cathartica* with conspecifics in its native range that could explain at least in part the invasiveness of *R*. *cathartica* in its introduced range. This indicates that the chances are slim of finding a specific soil-borne fungal pathogen with biocontrol potential.

Due to the difficulties surrounding currently studied agents and the low probability of finding additional potential agents, it has been decided that the project will be stopped. A publication summarizing main results will be prepared until 2013.

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## Annexes

**Annex 1.** Detection of '*Candidatus* Phytoplasma rhamni' in *Rhamnus* spp. and *Trichochermes walkeri* in 2010.

| | | Plants | | *Trichochermes walkeri* | |
|---|---|---|---|---|---|
| **Collection site** | **Date of collection** | **Plant species sampled (# samples = trees)** | ***'Ca.* Phytoplasma rhamni' analysis (# positive / # tested)** | ***Trichochermes walkeri* galls present (and collected)** | ***'Ca.* Phytoplasma rhamni' (# positive pulls / # tested)** |
| **Austria** | | | | | |
| A17 - Traiskirchen | 20 July 2010 | *Rhamnus cathartica* (5) | **2/5** | yes | 4/4 (18 nymphs) |
| A19 - Oberwaltersdorf | 21 July 2010 | *Rhamnus cathartica* (5) | **2/5** | yes | 2/4 (18 nymphs) |
| A48 - Truman | 21 July 2010 | *Rhamnus cathartica* (5) | **2/5** | no | - |
| A21 - Unterwaltersdorf | 21 July 2010 | *Rhamnus cathartica* (5) | **2/5** | yes | 4/4 (18 nymphs) |
| A25 - Purbach | 22 July 2010 | *Rhamnus cathartica* (5) | **2/5** | yes | 24/24 (103 nymphs) |
| A26 - St Margarethen | 21 July 2010 | *Rhamnus cathartica* (5) | **1/5** | no[a] | - |
| | | *Rhamnus saxatilis* (3) | **0/3** | no | - |
| **Switzerland** | | | | | |
| CH1 - Allondon | 4 August 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |
| CH3 - Satigny | 4 August 2010 | *Rhamnus cathartica* (2) | **0/2** | no[a] | - |
| | | *Frangula alnus* (5) | **0/5** | no | - |
| CH6 - Chatillon | 3 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| CH7 - La Combe | 3 August 2010 | *Rhamnus cathartica* (2) | **1/2** | yes | 5/5 (17 nymphs) |
| | 3 August 2010 | *Frangula alnus* (2) | **0/2** | no | - |
| CH10 - Courroux | 10 August 2010 | *Rhamnus cathartica* (3) | **0/3** | yes | 0/3 (9 nymphs) |
| CH11 - Delémont | 5 August 2010 | *Rhamnus cathartica* (3) | **1/3** | yes | 5/6 (19 nymphs) |

| | | Plants | | *Trichochermes walkeri* | |
|---|---|---|---|---|---|
| **Collection site** | **Date of collection** | **Plant species sampled (# samples = trees)** | **'*Ca.* Phytoplasma rhamni' analysis (# positive / # tested)** | ***Trichochermes walkeri* galls present (and collected)** | **'*Ca.* Phytoplasma rhamni' (# positive pulls / # tested)** |
| CH14 - Cheyres | 11 August 2010 | *Rhamnus cathartica* (5) | **5/5**[b] | yes | 24/24 (82 nymphs) |
| | | *Frangula alnus* (5) | **0/5** | no | - |
| CH17 - La Sauge | 11 August 2010 | *Rhamnus cathartica* (5) | **4/5** | yes | 26/27 (90 nymphs) |
| | | *Frangula alnus* (5) | **0/5** | no | - |
| CH19 - Vermes | 6 August 2010 | *Rhamnus cathartica* (5) | **0/5** | yes | 4/10 (2 adults + 29 nymphs) |
| | | *Rhamnus alpina* (5) | **0/5** | no | - |
| | | *Frangula alnus* (2) | **0/2** | no | - |
| CH30 - Soulce | 26 July 2010 | *Rhamnus cathartica* (5) | **3/5**[b] | yes | 10/13 (5 adults + 33 nymphs) |
| CH31 - Courcelon | 10 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| CH32 - Haute-Borne | 10 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| **Germany** | | | | | |
| D8 - Zienken | 28 July 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |
| D20 - Griessheim | 28 July 2010 | *Rhamnus cathartica* (5) | **2/5** | yes | 10/10 (2 adults + 28 nymphs) |
| D21 - Griessheim | 11 August 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |
| D22 - Griessheim | 11 August 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |
| D23 - Griessheim | 11 August 2010 | *Rhamnus cathartica* (4) | **1/4** | no[a] | - |
| **Serbia** | | | | | |
| Deliblatski pesak | 7 June 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |

| | | Plants | | *Trichochermes walkeri* | |
|---|---|---|---|---|---|
| **Collection site** | **Date of collection** | **Plant species sampled (# samples = trees)** | **'*Ca.* Phytoplasma rhamni' analysis (# positive / # tested)** | ***Trichochermes walkeri* galls present (and collected)** | **'*Ca.* Phytoplasma rhamni' (# positive pulls / # tested)** |
| | | *Rhamnus saxatilis* spp. *tinctorius* (5) | **0/5** | no | - |
| Sicevo | 8 June 2010 | *Rhamnus saxatilis* spp. *tinctorius* (5) | **0/5** | no | - |
| | | *Rhamnus rupestris* (5) | **0/5** | no | - |
| Rajac, East Serbia | 26 Jun 2010 | *Rhamnus cathartica* (5) | **1/5** | no[a] | - |
| Deliblatski pesak, Susara | 30 July 2010 | *Rhamnus cathartica* (5) | **1/12** | yes | 3/11 (11 adults + 1 nymph) |
| Deliblatski pesak, Susara | 30 July 2010 | *Rhamnus saxatilis* spp. *tinctorius* (5) | **0/5** | no | - |
| Deliblatski pesak, exit | 30 July 2010 | *Rhamnus cathartica* (5) | **0/5** | no[a] | - |
| Mitrovac na Tari | 1 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| Tara | 1 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| Cerovica, kanjon iza Knjazevca | 13 August 2010 | *Rhamnus cathartica* (5) | **0/5** | no | - |
| Beranje | 8 Sept. 2010 | *Rhamnus cathartica* (5) | **1/5** | no | - |
| Rajac | 9 Sept. 2010 | *Rhamnus cathartica* (4) | **3/4** | no[a] | - |
| **Montenegro** | | | | | |
| Kolasin | 17 August 2010 | *Rhamnus cathartica* (2) | **0/2** | no | - |
| Kolasin | 17 August 2010 | *Rhamnus alpina* (5) | **0/5** | no | - |
| Nudo | 18 August 2010 | *Rhamnus rupestris* (5) | **0/5** | no | - |

[a] *Trichochermes walkeri* was recorded in previous years.

[b] Phytoplasma-positive samples of *T. walkeri* were collected in 2009.

**Annex 2.** Results of a mixed-effect model on the influence of site (La Sauge vs Cheyre), soil (*Rhamnus cathartica* area vs *R. cathartica*-free area) and sterilization (non-sterile vs. sterile soil) on seedling emergence and seedling growth of *Rhamnus cathartica.* Significant *P*-values are in bold; ndf and ddf denote numerator and denominator degrees of freedom, respectively. Mineral N (nitrogen) values and plant biomass data were $\log_{10}$-transformed prior to analysis.

| **Tray Data:** | | **Mineral N** | | **Percentage seedling emergence** | |
|---|---|---|---|---|---|
| | ndf,ddf | F | *P* | F | *P* |
| Site | 1,4 | 9.60 | **0.036** | 7.68 | **0.050** |
| Soil | 1,8 | 0.10 | 0.764 | 0.04 | 0.849 |
| Sterile | 1,16 | 5.50 | **0.032** | 24.02 | **<0.001** |
| Site × Soil | 1,8 | 1.56 | 0.246 | 1.34 | 0.281 |
| Site × Sterile | 1,16 | 1.91 | 0.186 | 4.29 | 0.055 |
| Soil × Sterile | 1,16 | 0.23 | 0.639 | 0.09 | 0.764 |
| Site × Soil × Sterile | 1,16 | 0.55 | 0.468 | 0.85 | 0.371 |

| **Plant Data:** | | **Days to seedling emergence** | | **Plant height** | | **No. of leaves** | |
|---|---|---|---|---|---|---|---|
| | ndf,ddf | F | *P* | F | *P* | F | *P* |
| Site | 1,4 | 0.49 | 0.522 | 1.62 | 0.272 | 2.24 | 0.209 |
| Soil | 1,8 | 2.23 | 0.174 | 2.57 | 0.148 | 3.94 | 0.082 |
| Sterile | 1,16 | 3.55 | 0.078 | 9.69 | **0.007** | 39.15 | **<0.001** |
| Site × Soil | 1,8 | 0.25 | 0.629 | 0.00 | 0.949 | 0.00 | 0.997 |
| Site × Sterile | 1,16 | 0.00 | 0.967 | 11.73 | **0.004** | 6.59 | **0.021** |
| Soil × Sterile | 1,16 | 0.29 | 0.598 | 1.27 | 0.276 | 0.42 | 0.525 |
| Site × Soil × Sterile | 1,16 | 1.32 | 0.268 | 0.74 | 0.404 | 0.63 | 0.440 |

| | | **Total biomass** | | **Belowground biomass** | | **Aboveground biomass** | |
|---|---|---|---|---|---|---|---|
| | ndf,ddf | F | *P* | F | *P* | F | *P* |
| Site | 1,4 | 0.00 | 0.961 | 0.04 | 0.854 | 0.07 | 0.803 |
| Soil | 1,8 | 3.55 | 0.096 | 3.88 | 0.084 | 2.72 | 0.138 |
| Sterile | 1,16 | 17.37 | **<0.001** | 6.67 | **0.020** | 27.53 | **<0.001** |
| Site × Soil | 1,8 | 0.06 | 0.818 | 0.13 | 0.731 | 0.00 | 0.981 |
| Site × Sterile | 1,16 | 1.09 | 0.312 | 0.35 | 0.562 | 5.58 | **0.031** |
| Soil × Sterile | 1,16 | 1.68 | 0.214 | 3.09 | 0.098 | 0.52 | 0.482 |
| Site × Soil × Sterile | 1,16 | 0.12 | 0.733 | 0.00 | 0.955 | 0.39 | 0.542 |

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