The French walnut improvement program: preliminary investigations

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Abstract

With 36,000 tons of in-shell walnuts produced in 2016, France is in 9th world position among major producers. Walnut orchards increased by almost 20% between 2000 and 2010, and walnut is the most important crop other than apple. Nevertheless, the varietal choice does not appear to be adapted to the new future constraints and the new French walnut improvement program, led by the Ctifl, has to take into account the global warming context and the reduced use of plant protection products. In this way, two preliminary studies have been conducted regarding the effect of climate change on walnut phenology under French climate conditions, and concerning an emerging disease in French walnut orchards, a form of anthracnose, due to *Colletotrichum acutatum*. On the one hand, as regards phenology aspect, chronological data have been collected for the two most produced cultivars in France thanks to the late Eric Germain, former head of breeding program at INRA of Bordeaux from 1977 to 2007 and thanks to the work of the two French walnut experimental stations of Creysse and SENuRA. A significant advance in bud break date and male and female flowering dates was found. On the other hand, as concerns the emerging disease, a qualitative detached leaf assay (absence or presence of necrosis) have been carried out in order to quickly find out which accessions of the French germplasm (including 220 accessions of *J. regia*, the cultivated walnut tree, and 39 accessions of related species) are tolerant. Results show that, after 7 days of spores inoculation on detached leaflets, all *J. regia* accessions have specific symptoms of anthracnose due to *C. acutatum* (black to brown spots with orange acervules) while two related species, *J. cinerea* and *J. sieboldiana*, do not present any symptom, revealing a potential tolerance to *C. acutatum*.

Keywords:

Juglans regia L., French walnut improvement program, chronological data, phenology, *Colletotrichum acutatum*, detached leaflet assay

INTRODUCTION

If the main goals of walnut breeding in France are a lateral bearing habit, an increased yield, a larger fruit size, a light color, an organoleptic quality, a thicker shell, an ease of cracking, and a tolerance to walnut blight (Bernard et al., 2018), two important traits must be considered: a well adaptation to French climatic conditions, with a late budbreak due to late spring frosts, and the tolerance to *Colletotrichum acutatum*, an emerging pathogen.

Colletotrichum spp. is an ascomycete genus of fungi ranked among a 'Top 10' fungal plant pathogen list based on scientific/economic importance (Dean et al., 2012). Among the species, *C. acutatum* J.H. Simmonds (sexual stage: *Glomerella acutata*) is a widespread complex of 29 species (Damm et al., 2012) responsible for a form of anthracnose and has a wide range of host crops worldwide such as apple (Jones et al., 1996), strawberry (Xie et al., 2010) and almond (Förster & Adaskaveg, 1999). In French walnut orchards, *Colletotrichum* spp. is known to have emerged in 1998-2000 from the observation of walnuts with symptoms of Brown Apical Necrosis (Belisario et al., 2002) and it has been characterized as necrotrophic in walnut in France since 2008. Symptoms classically include the appearance of black to brown spots with orange acervules, which often leads to a completely necrotic walnut that falls prematurely. There is currently no effective mean of control to prevent this pathogen and one of the levers would be the search for tolerant genotypes.

In parallel, climate change has impacted plant phenology in last decades, particularly in favor of an advance in flowering dates (Chmielewski et al., 2001; Menzel et al., 2006; Jochner et al., 2015). If late spring frosts are known to be damaging in French walnut, it was also shown that early frost damages could negatively influence fruit yield in the following year (Charrier et al., 2017). It is therefore important to assess the effect of environment on the phenology of the main cultivars currently produced in France.

Because *C. acutatum* attack and global warming can both lead to loss of French walnut production, the objectives of this study are, on the one hand, to determine which available accessions have or do not have necrosis caused by an isolate of *C. acutatum* using an *in vitro* detached leaflet assay (DLA) with observation of symptoms at 7 days after inoculation (DAI) and on the other hand, to study the budbreak and the male/female flowering dates of 'Franquette' and 'Lara' cultivars of the last decades using chronological data.

MATERIALS AND METHODS

Evaluation of the tolerance of French germplasm to *Colletotrichum acutatum* using detached leaf assay

Fungal isolation: Several walnuts that showed typical symptoms of anthracnose disease caused by *C. acutatum* were collected in spring 2016 from trees in arboriculture experimental unit of

INRA in Toulenne, near Bordeaux (France). After cleaning with water and 70% ethanol, several small square-shaped pieces of necrotic walnut husks were cut with a scalpel and placed on Potato Dextrose Agar (PDA) medium with streptomycin sulfate to inhibit growth of bacteria. Successive subculturing was performed to obtain several purified colonies.

Plant materials collection and surface sterility obtaining: 220 *J. regia* accessions and 39 of related species (including *Rhysocaryon* section (*J. nigra*, *J. hindsii*, *J. microcarpa*, *J. major*, *J. mollis*) and *Cardiocaryon* section (*J. cinerea*, *J. sieboldiana*, *J. cathayensis*, *J. mansdhurica*)) were screened. All accessions are grafted trees located in the arboriculture experimental unit in Toulenne (latitude 44°34'37.442''N – longitude 0°16'51.48''O), near Bordeaux (France). Five mature and healthy leaflets from compound leaves randomly taken from all over the tree were hand-collected for each accession, from May to July 2017 to globally respect the age of leaflets according to budbreak dates. Surface sterility was obtained by a rinse in 70% ethanol for 50 seconds and three consecutive rinses in sterile distilled water (SDW) for 50 seconds. Each of the five leaflets is placed, on laminar flow hood, in an annotated Petri dish with adaxial surface in contact with four layers of sterile blotting paper moistened with SDW, in order to maintain leaflet turgidity with free stomata and to prevent evaporation until symptoms are observed.

Leaflets inoculation and growing conditions: A solution of germinated spores from *C. acutatum* isolate with a concentration of 1.10^5 spores/mL is produced thanks to a hemocytometer. One leaflet by accession, chosen as control, is wounded with a sterile needle and inoculated with a 10 µL drop of SDW. The four other leaves are equally wounded but inoculated with a 10 µL drop of the solution of spores. Four inoculation points are performed, two on the blade and two on the central rib. All the Petri dishes are sealed with Parafilm during transport to the climatic chamber. Growing conditions, trade-off between the host and the pathogen, are as follows: temperature of 23±1°C/day and 18±1°C/night, artificial light for 16h/day and 8h/night, and 80% humidity rate.

Symptoms observation and image processing: Symptoms are recorded 7 DAI with image capture. Image processing is then realized using APS Assess 2.0 software (Lamari, 2008) to measure the size of the necrosis.

Fungal identification: Koch's postulates could not be verified on all the 259 accessions. Thus, in order to prove the presence of *C. acutatum* in the typical necrosis, a molecular identification was performed on a randomly selected accession by PCR using specific primers based on the ITS region to *C. acutatum* (CaInt2: 5′-GGGGAAGCCTCTCGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) and to *C. gloeosporioides* (CgInt: 5′-GGCCTCCCGCCTCCGGGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) (Liu et al., 2012), a related species often confused with and also found to be pathogenic in French walnut orchards. The PCR reactions were carried

out using DNA samples extracted with the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions for both plants and fungi samples. The amplification reactions were performed in 23 µL with 2 µL DNA matrix, 5 µL 5X buffer, 0.05 µL dNTP, 0.25 µL each primer and 0.2 µL Taq polymerase. PCR conditions were as follows: denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 59°C for 2 min and 72°C for 2 min, and final extension at 72°C for 10 min. The PCR products were checked on 1% agarose gel by electrophoresis at 130V for 25 min. All PCR experiments were repeated two times with identical results.

Study of budbreak date and male/female flowering dates using historical data

Plant materials and phenotypical traits observations: Phenotypical data have been collected for 'Franquette' and 'Lara' cultivars, for a period from 1989 to 2016, and on three different sites: arboriculture experimental unit in Toulenne (latitude 44°34'37.442''N – longitude 0°16'51.48''O), walnut experimental station of Creysse (latitude 44°53'16.529''N – longitude 1°36'23.838''E) and walnut experimental station of SENuRA (latitude 45°8'38.565''N – longitude 5°17'34.323''E). Budbreak dates and male/female flowering dates have been observed according to IPGRI descriptors for walnut (IPGRI, 1994) improved by the late Eric Germain, former head of breeding program at INRA of Bordeaux from 1977 to 2007 (Table 1).

Table 1. Walnut descriptors

Phenological trait observed	Description	Unit	Picture
Date of bud break	When over 50% of terminal buds have enlarged and bud scales have split exposing the green of the leaves inside	In the format DDMMYYYY	
Peak male bloom date	When maximum pollen shedding occurs	In the format DDMMYYYY	
Peak female bloom date	Date of maximum pistillate flower receptivity	In the format DDMMYYYY	

RESULTS AND DISCUSSION

Evaluation of the tolerance of French germplasm to *Colletotrichum acutatum* using detached leaf assay

Fungal identification: H 109-3 accession was chosen randomly to perform fungal molecular identification. PCR products show the presence of *C. acutatum* and the absence of *C. gloeosporioides* in necrosis of H 109-3 samples (Fig. 1).

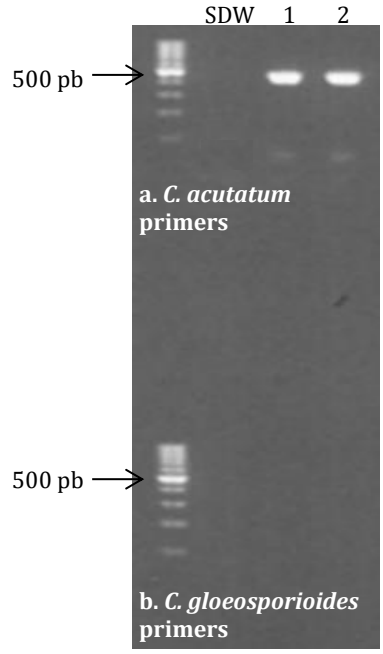


Figure 1. Molecular identification of *C. acutatum* on randomly selected H 109-3 accession necrosis. 1 + 2: necrosis samples of H 109-3; SDW: sterilised distilled water. *C. acutatum* is present in H109-3 necrosis as revealed with *C. acutatum* primers (a) and *C. gloeosporioides* is absent in H109-3 necrosis as revealed with *C. gloeosporioides* primers (b).

Symptoms observation and tolerant accessions: Inoculation of detached walnut leaflets with a solution of spores of *C. acutatum* resulted, for susceptible accession H 109-3, in a clearly defined necrosis which rapidly spread within few days (Fig. 2). If literature does not prove the presence of symptoms on leaflets in French walnut orchards, this work firstly permitted to show that *C. acutatum* is able to induce necrosis on detached leaflets within 7 DAI whereas the symptoms are mostly observed on fruits. The primary objective of this detached leaflet assay was to perform a quantitative measure of necrosis according to genotypes. However, statistical analysis performed on the repeatability of the test (Friedman test) showed that it was not able to be quantitative but only qualitative with presence or absence of necrosis. Among the 259 accessions tested, all the *J. regia* accessions showed a presence of necrosis at 7 DAI. Nevertheless, seven accessions among related species showed an absence of necrosis at 7 DAI. There were the four *J. cinerea* accessions (CN 13-3, CN 1-9, CN 19-2 and CN 8-1) and the three *J. sieboldiana* accessions (SB 1-30, SB 24-1 and SB 6-9) (Fig. 2). These *Juglans* species found to be

tolerant to *C. acutatum* on our detached leaflet assay, *J. cinerea* and *J. sieboldiana*, seem to be also tolerant to other diseases according to literature. Indeed, in Belisario et al., 1999, *J. cinerea* and *J. sieboldiana* were reported to be the most resistant species to bacterial blight caused by *Xanthomonas campestris* pv. *juglandis*. In another work of Belisario et al., 2008, *J. cinerea* and *J. sieboldiana* were also reported to be the most resistant species to *Gnomonia leptostyla*, the other agent responsible for another anthracnose form. In Pollegioni et al., all *J. regia* accessions were found to be susceptible to *G. leptostyla*. They also found an effect of leaf age, as we noted in our work. In Tsiantos et al., 2007, some *J. regia* cultivars ('Amigo', 'Ashley', 'Serr', 'Grand Jean') were less susceptible to *C. acutatum* but in our case, all *J. regia* accessions could have developed necrosis at 7 DAI. This evidence of global susceptibility of *J. regia* to pathogens was also found in Arnaudov et al., 2015. Indeed, even if sensibility levels do exist, all genotypes showed symptoms of *X. arboricola* pv. *juglandis* and *G. leptostyla*. If there is little information available on the tolerance level of walnut cultivars to *C. acutatum*, this first germplasm screening suggests that the sources of strict tolerance will not be found in cultivated walnut accessions but in related species of *Juglans* genus, making it difficult in terms of genetic improvement by conventional techniques such as breeding. In addition, investigation of the tolerance level of the accessions with a detached leaflet assay was chosen, but if there are advantages such as symptoms observation on many individuals at the same time (Mo et al., 2007) and space saving, there are also many drawbacks such as a not always accurate representation of natural conditions and sometimes, an absence of correlation with orchard trials.

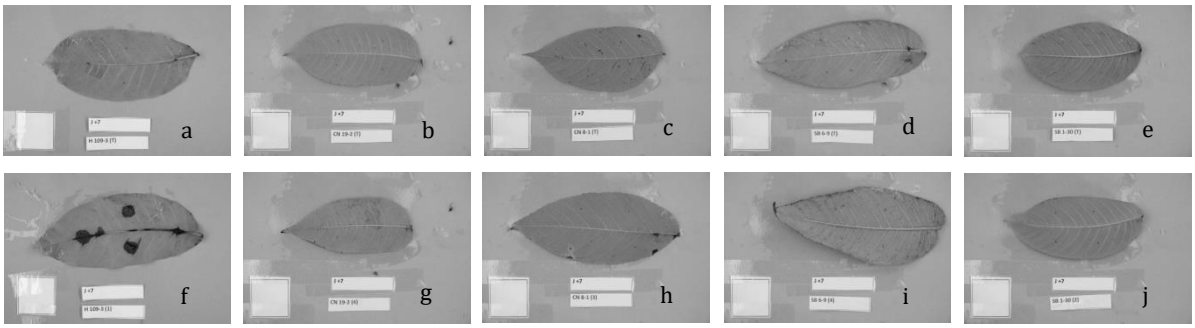
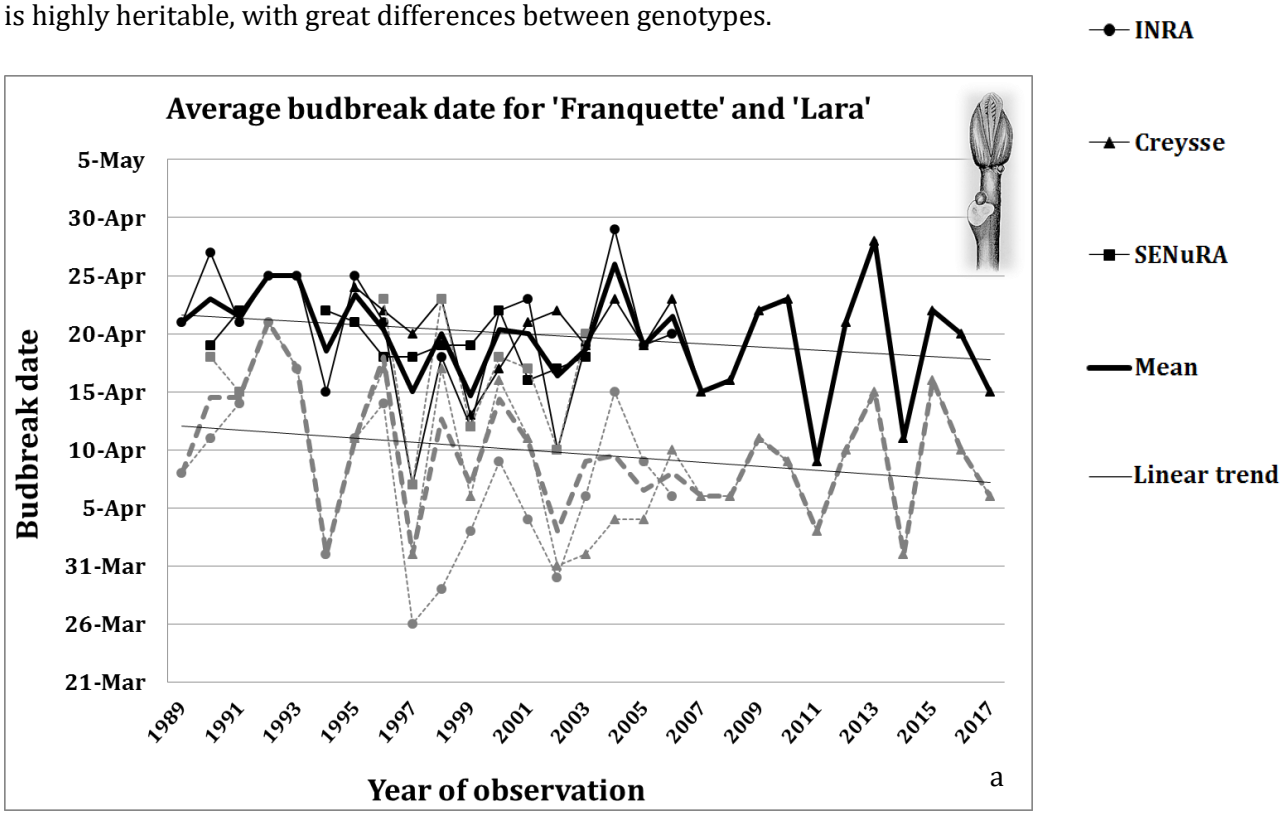


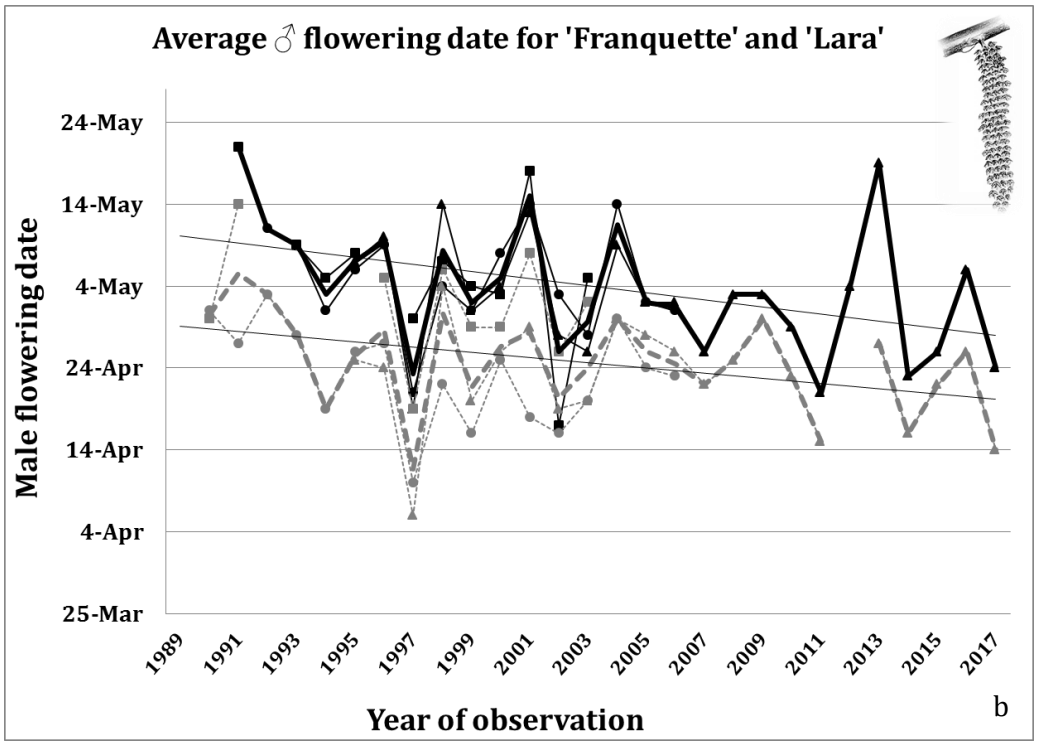
Figure 2. Observation of symptoms caused by *C. acutatum* at 7 DAI. For control leaflets, at the top, there is no evolution in the size of necrosis due to needle wound and SDW drop at 7 DAI (a, b, c, d and e). For infected leaflets, at the bottom, H 109-3 accession (f) shows necrosis at 7 DAI with characteristic symptoms: black to brown spots with orange acervules. On the contrary, for two accessions of *J. cinerea*: CN 19-2 (g) and CN 8-1 (h) and two accessions of *J. sieboldiana*: SB 6-9 (i) and SB 1-30 (j), there is no evidence of necrosis development at 7 DAI.

Study of budbreak date and male/female flowering dates using chronological data

Over the years, there is a tendency to an advance of phenology in 'Franquette' and 'Lara' cultivars under French climatic conditions for budbreak date and for male/female flowering dates (Fig. 3). Data are quite comparable for the three experimental stations and they follow the same fluctuations between the two cultivars. As concerns the budbreak graph, there is two years when the advance of the date was very significant: 1994 and 1997. In parallel, in France, the months of March 1994 and 1997 were recorded as being the warmer of the last thirty years, with that of 2017. In March 1994 and 1997, the mean temperature was 10.7°C against 8.7°C usually (Météo France data). On the contrary, winter 2013 vas recorded as colder than the average and there is a delay in phenology in 2013. Overall, the same results are found regarding the male flowering and the female flowering dates. It seems that the phenology of walnut tree is sensitive and directly related to the weather conditions preceding the events, even if phenology is highly heritable, with great differences between genotypes.



a



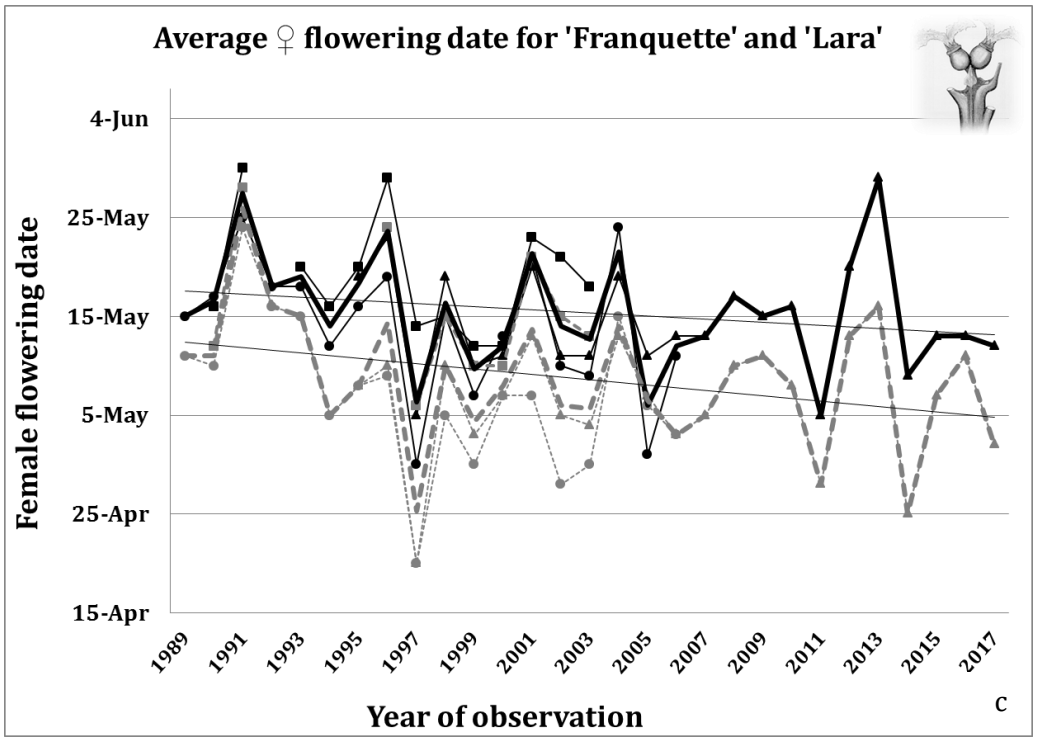


Figure 3. Phenological traits observed for 'Franquette' (solid and black line) and 'Lara' (dotted and grey line) cultivars from 1989 to 2017 on 3 experimental sites (INRA, Creysse and SENuRA). (a) Budbreak date, (b) Male flowering date, (c) Female flowering date.

CONCLUSION

One of the major challenges of the French walnut improvement program is to determine the most moderate levels of susceptibility to *C. acutatum* within the germplasm available. Thus, a

number of detached fruits assays will be set up during summer 2018 on contrasting genotypes to obtain a repeatable quantitative protocol. This protocol could then be tested on the *J. regia* collection, to determine the less-susceptible accessions. In parallel, the germplasm collection will be observed regarding phenology. With pathological and phenological characterization of the collection, and with genotyping thanks to the help of the 600K SNP Affymetrix® array already initiated, we will have elements to find out genomics regions involved with those two traits by performing a Genome-Wide Association Study. This knowledge could be useful for the choice of future parents for the new breeding program.

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Literature Cited

Arnaudov, V., Gandev, S., Dimova, M. (2015). Susceptibility of Some Walnut Cultivars to *Gnomonia leptostyla* and *Xanthomonas arboricola* pv. *juglandis* in Bulgaria. Agroznanje. *15*, 41–54.

Belisario, A., Maccaroni, M., Corazza, L., Balmas, V., Valier, A. (2002). Occurrence and Etiology of Brown Apical Necrosis on Persian (English) Walnut Fruit. Plant Dis. *86*, 599–602.

Belisario, A., Scotton, M., Santori, A., Onofri, S. (2008). Variability in the Italian population of *Gnomonia leptostyla*, homothallism and resistance of *Juglans* species to anthracnose. For Pathol. *38*, 129–145.

Belisario, A., Zoina, A., Pezza, L., Luongo, L. (1999). Susceptibility of species of *Juglans* to pathovars of *Xanthomonas campestris*. For Pathol. *29*, 75–80.

Bernard, A., Lheureux, F, Dirlewanger, E. (2018). Walnut: past and future of genetic improvement. Tree Genetics & Genomes. *14*, 1.

Charrier, G., Chuine, I., Bonhomme, M., Améglio, T. (2017). Assessing frost damages using dynamic models in walnut trees: exposure rather than vulnerability controls frost risks. Plant Cell Environ.

Chmielewski, F.M., Rötzer, T. (2001). Response of tree phenology to climate change across Europe. Agric For Meteorol. *108*, 101–112.

Damm, U., Cannon, P.F., Woudenberg, J.H.C., Crous, P.W. (2012). The *Colletotrichum acutatum* species complex. Stud Mycol. *73*, 37–113.

Dean, R., Van Kan, J.A.L., Pretorius, Z.A., Hammond-Kosack, K.E., Di Pietro, A., Spanu, P.D., et al. (2012). The Top 10 fungal pathogens in molecular plant pathology. Mol Plant Pathol. *13*, 414–430.

Förster, H., Adaskaveg, J.E. (1999). Identification of Subpopulations of *Colletotrichum acutatum* and Epidemiology of Almond Anthracnose in California. Phytopathology. *89*, 1056–1065.

Jochner, S., Menzel, A. (2015). Does flower phenology mirror the slowdown of global warming? Ecol Evol. *5*, 2284–2295.

Jones, A.L., Ehret, G.R., Meyer, M.P., Shane, W.W. (1996). Occurrence of Bitter Rot on Apple in Michigan. Plant Dis. *80*, 1294–1297.

Lamari, L. Assess 2.0: image analysis software for plant disease quantification. (2008). St. Paul, MN: American Phytopathological Society.

Liu, B., Louws, F.J., Sutton, T.B., Correll, J.C. (2012). A rapid qualitative molecular method for the identification of *Colletotrichum acutatum* and *C. gloeosporioides*. Eur J Plant Pathol. *132*, 593–607.

Menzel, A., Sparks, T.H., Estrella, N., Koch, E., Aasa, A., Ahas, R., et al. (2006). European phenological response to climate change matches the warming pattern. Glob Change Biol. *12*, 1969–1976.

Mo, J.Y., Navi, S.S., Li, X., Guo, T., Yang, X.B. (2007). Detached Leaf Assay: A Rapid Screening Technique to Study Foliar Diseases of Soybean. Plant Pathology Presentations and Posters.

Pollegioni, P., Van der Linden, G., Belisario, A., Gras, M., Anselmi, N., Olimpieri, I., et al. (2012). Mechanisms governing the responses to anthracnose pathogen in *Juglans* spp. J Biotechnol. *159*, 251–264.

Tsiantos, J., Vagelas, I.K., Rumbos, C.I., Chatzaki, A., Rouskas, D., Gravanis, F.T. (2007). Evaluation of resistance of cultivated walnut varieties and selections *to Xanthomonas arboricola* pv. *juglandis* in Greece. Cost 873 WG Manag Comm Meet. Athens, Greece.

Xie, L., Zhang, J., Wan, Y., Hu, D. (2010). Identification of *Colletotrichum* spp. isolated from strawberry in Zhejiang Province and Shanghai City, China. J Zhejiang Univ Sci B. *11*, 61–70.