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Backcross, transgenic, or both? Using logic models and data to decide on effective paths to evolutionary rescue of American chestnut

Author(s):

Dr. Jared Westbrook, The American Chestnut Foundation, 50 North Merrimon Avenue Suite 115, Asheville, North Carolina, 28804, United States of America; jared.westbrook@acf.org (presenting author)
Dr. Vasiliy Lakoba, The American Chestnut Foundation, 29010 Hawthorne Dr 2928, Meadowview, Virginia, 24361, United States of America; vasiliy.lakoba@acf.org (co-author)
Sara Fitzsimmons, The American Chestnut Foundation, Pennsylvania State University, 108 Business 206 Forest Resources Lab, University Park Pennsylvania 16802, United States of America; sara.fitzsimmons@acf.org (co-author)
Alexander Sandercock, Virginia Tech University, 451 Latham Hall, Blacksburg Virginia 24061, United States of America; amsand@vt.edu (co-author)
Prof. Jason Holliday, Virginia Tech University, 451 Latham Hall, Blacksburg Virgiia 24061, United States of America; jah1@vt.edu (co-author)

Abstract:

Restoration of American chestnut depends on producing a founder population that has adequate disease resistance, forest competitiveness, and genetic diversity to adapt to a large natural range and a changing climate. Backcross breeding to introduce chestnut blight resistance from Chinese chestnut has been challenging because blight resistance is genetically complex and there is a strong tradeoff between blight resistance and American chestnut ancestry. To date, the most promising approach to enhance blight resistance has been the transgenic insertion of the oxalate oxidase (OxO) gene from wheat, which detoxifies oxalic acid produced by the blight fungus and significantly reduces the severity of stem cankers. U.S. federal regulators are expected decide whether to grant approval to distribute

progeny of the Darling 58 variety containing OxO in 2023. Using speed breeding techniques, The American Chestnut Foundation (TACF) has begun introgressing OxO into a range wide sample of American chestnut individuals with the aim of representing > 95% of the climate adaptive genetic diversity in *Castanea dentata*. We have also begun breeding Darling 58 progeny with backcross trees that inherited resistance to phytophthora root rot (PRR) to generate dual resistance trees. Some landowners prefer non-transgenic trees, and hence TACF is pursuing controlled pollinations between the most blight resistant backcross selections. Methods to compare the efficacy of these approaches will be discussed including seedling assays to obtain early estimates of blight and PRR resistance, orchard trials to assess disease resistance and growth over the long term, and forest trials to assess competitiveness and ecological interactions.

Keywords: *Cryphonectria parasitica, Phytophthora cinnamomi*, genomic selection, marker assisted introgression, local adaptation

Chestnut trees (*Castanea sativa* Mill.) for climate change

Author(s):

Alejandro Solla, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; asolla@unex.es (presenting author)
F. Javier Dorado, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; fdoradoreyes@unex.es (co-author)
Rubén González, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; rubenglezglez@unex.es (co-author)
Luz Belén Giraldo-Chaves, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; lbgch_1308@hotmail.es (co-author)
Elena Cubera, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; ecubera@unex.es (co-author)
Gregorio Rocha, Faculty of Forestry, Universidad de Extremadura, Avenida Virgen del Puerto 2, 10600 Plasencia, Spain; gregorio@unex.es (co-author)
César Martín, Viveros Forestales la Dehesa SL, Calle Gabriel y Galán 38, 10672 Valdeovispo, Spain; info@viverosladehesa.com (co-author)
Efrén Martín, Agrocastanea SL, Fernán Pérez de Monroy 2, 10490 Valverde de la Vera, Spain; arbole14@gmail.com (co-author)
Beatriz Cuenca, TRAGSA, Maceda Nursery, Carretera de Maceda a Baldrei km 2, 32700 Maceda, Ourense, Spain; bcuenca@tragsa.es (co-author)
José Luis del Pozo, Servicio de Ordenación y Gestión Forestal, Consej. Industria, Energía y Medio Ambiente, Junta de Extremadura, 06800 Mérida, Spain; joseluis.pozo@juntaex.es (co-author)

Felipe Pérez, Dirección General de la Biodiversidad , Bosques y Desertificación, MITECO, Madrid, Spain; fperez@miteco.es (co-author)
M. ngela Martín, Department of Genetics, ETSIAM, University of Córdoba, 14071 Córdoba, Spain; angela.martin@uco.es (co-author)

Abstract:

As climate changes, getting warmer and drier in countries within the Mediterranean Basin, selection of new chestnut trees to be planted in orchards and forests is needed. In 2015, the Faculty of Forestry at Universidad de Extremadura (Spain) initiated a breeding program aimed to select *Castanea sativa* genotypes tolerant to global change. This program is currently supported by chestnut growers, regional funds and the Ministry for the Ecological Transition. Nuts from natural *C. sativa* stands located in Andalusia (Constantina and Paterna del Río) and Extremadura (Hervás and Valle de Matamoros) were collected and sown under a randomized block design. Circa 300 one-year-old seedlings per population were challenged with *Phytophthora cinnamomi* for two years. The eight most tolerant plants were selected, established *in vitro* and micropropagated. Clonal replicates were subjected during two years to drought, heat, waterlogging, and *P. cinnamomi* conditions (n=8). Commercial 111-1, 7521, 2671 and 90.044 clones, tolerant to *P. cinnamomi*, were included in the tests as controls. Two *C. sativa* genotypes, 'Paterna del Río 18' and 'Valle de Matamoros 1', showed resistance to *P. cinnamomi*, high levels of tolerance to drought and heat stress, and acceptable levels of tolerance to waterlogging. SSR markers confirmed that both clones were *C. sativa*. Replicates have been planted in two different experimental field plots in order to assess plant performance and adaptation (n=10). 'Paterna del Río 18' and 'Valle de Matamoros 1' will be registered soon, being the first chestnut clones tolerant to climate change deployed in Spain.

Keywords: tree breeding, climate change, ink disease

Performance of ten elite Chinese Chestnut Cultivars in Replicated Trial over 16 years in Central Missouri

Author(s):

Dr. Nicholas Meier, 10 Research Center Rd, New Franklin MO 65274, United States of America; namvf6@missouri.edu (presenting author)
Prof. Michael Gold, 450 Hatchery Rd, N. Kingstown RI 02852, United States of America; GoldM@missouri.edu (co-author)
Assist. Prof. Ronald Revord, 303Q Anheuser-Busch Natural Resources Build,

Columbia MO 65211, United States of America; r.revord@missouri.edu (co-author)

Abstract:

Chinese chestnut is an emerging specialty crop in the Midwestern USA. Long term evaluation of elite Chinese chestnut cultivars is limited in the USA, leading to confusion among growers regarding cultivar selection. To inform Mid-Missouri growers more accurately, 5 complete replicated blocks (7.6x7.6 m spacing) of 10 promising cultivars of predominately *Castanea mollissima* ancestry were field grafted onto *C. mollissima* rootstock on deep, fertile, well drained loess soil of the Missouri River hills in 1999 at the Horticultural and Agroforestry Research Center (New Franklin, Missouri, USA). Yield (kg/tree) and kernel weights (g) were collected 11 times over a period of 16 years (2007-2011, 2015, 2017, and 2019-2022). Additional kernel quality (17) and phenological parameters (11) were evaluated during 2020-2022. 'Qing' produced the highest yields during 9 of the 11 years that data was collected. 'Payne' produced the highest yields in 2020 and 2021. The highest average single season yield was achieved by 'Payne' in 2021 averaging 45.5 kg/tree. 30-nut samples were evaluated for weight, dimension, percent defect, ease of peeling, pellicle thickness, and flesh color to gauge overall quality. Nut weight (g) varied year to year and was influenced by moisture availability and crop load. Data collected over a 16-year period (N=11) revealed nut weights averaging between 10.1-15.4 g, with 'Peach' the heaviest and 'Perry' the lightest. Total variation (%CV) for nut weight (g) was lowest for 'Mossbarger' (8.2%) and 'Peach' (8.6%) and highest for Qing (23.6%). Variation in phenology, harvest time, harvest window, percent nut defect, and other quality parameters were also significant amongst cultivars. This cultivar trial remains under evaluation for yield, nut quality, post-harvest spoilage, and pest/disease incidence. A new trial with 2 to 5 replications of 122 cultivars/advanced selections was established in 2022 at UMCA to evaluate additional germplasm, the top performing cultivars from the original trial are included as commercial checks.

Keywords: *Castanea mollissima*, Chinese Chestnut, Replicated Complete Block, Cultivar Trial, Yield, Nut Quality, Missouri, USA

Conservation of the Ozark chinquapin by applying biotechnology developed for American chestnut

Author(s):

Dr. Patrícia Fernandes, SUNY-ESF, 1 Forestry Drive, 13210 Syracuse, NY, United States of America; pmoraisf@esf.edu (presenting author)
Grant Dollmann, SUNY-ESF, Syracuse, NY, United States of America; gmdollma@syr.edu (co-author)
Hannah Pilkey, SUNY-ESF, Syracuse, NY, United States of America; hcpilkey@esf.edu (co-author)
William Powell, SUNY-ESF, Syracuse, NY, United States of America; wapowell@ESF.EDU (co-author)
Andrew Newhouse, SUNY-ESF, Syracuse, NY, United States of America; aenewhou@esf.edu (co-author)

Abstract:

The necrotrophic pathogen *Cryphonectria parasitica* (causal agent of chestnut blight) left the North American chestnut species functionally extinct in the 20th century. The American chestnut (*Castanea dentata*)-*C. parasitica* interaction has been studied for decades, and several field and laboratory techniques have been optimized to help reintroduce this tree. A blight-tolerant American chestnut (Darling 58) was developed by adding a gene from wheat encoding for a detoxifying enzyme, oxalate oxidase (*OxO*), to counter the main virulence factor of the pathogen. With the imminent deregulation of Darling 58 by the U.S. regulatory agencies, a next logical step is to apply the gathered knowledge to other closely related species impacted by the chestnut blight, such as the Ozark chinquapin (*Castanea ozarkensis*). We are using two approaches in parallel: backcross breeding and genetic transformation. Darling 58 pollen is being used as source of blight tolerance to introgress the OxO gene into the Ozark chinquapin: crosses were performed in 2021/2022 and transgenic F1 hybrid embryos were isolated for in vitro culture establishment. These are currently being propagated. Plants produced in vitro will be placed under high light treatments for rapid pollen production, which will be used to backcross these F1 hybrids to full Ozark chinquapin parent trees and begin the process of restoring the species' characteristics. The direct genetic transformation of Ozark chinquapin somatic embryos with *OxO* is ongoing parallel to breeding. Our in vitro production pipeline was tested in wild-type *C. ozarkensis* prior to transformations. All steps from embryo multiplication and regeneration to shoot rooting were successful. The applicability of the American chestnut's *Agrobacterium*-mediated transformation protocol was first confirmed by obtaining *C. ozarkensis* somatic embryos expressing a reporter gene, and the first *OxO* transformants were recently obtained. These results show great promise for meeting the challenge of developing a blight-tolerant Ozark chinquapin. Other closely related forest tree species under threat might also benefit from methods developed for the American chestnut.

Keywords: *Agrobacterium*-mediated transformation; *Castanea dentata; Castanea ozarkensis*; chestnut blight; oxalate oxidase

Application of CRISPR/cas9 technology to chestnut breeding

Author(s):

Vera Pavese, DISAFA - University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco TO, Italy; vera.pavese@unito.it (co-author)
Andrea Moglia, DISAFA - University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco TO, Italy; andrea.moglia@unito.it (co-author)
Elena Corredoira, Misión Biológica de Galicia - CSIC, Avd. Vigo, sn , 15705 Santiago de Compostela, Spain; elenac@mbg.csic.es (co-author)
Anna Maria Milani, DISAFA - University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco TO, Italy; annamaria.milani@unito.it (co-author)
Marina Teresa Martinez, Misión Biológica de Galicia - CSIC, Avd. Vigo, sn, 15705 Santiago de Compostela, Spain; temar@mbg.csic.es (co-author)
Daniela Torello Marinoni, DISAFA - University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco TO, Italy; daniela.marinoni@unito.it (co-author)
Prof. Roberto Botta, DISAFA - University of Torino, Largo Paolo Braccini 2, 10095 Grugliasco TO, Italy; roberto.botta@unito.it (presenting author)

Abstract:

The CRISPR/Cas9 technology represents an innovative tool for breeding. It is a highly precise technique that can perform target mutation on specific genes, leading to complete gene deactivation. This technology is an interesting way to accelerate plant breeding but it is still poorly applied to woody species due to the low transformation efficiency and high regeneration recalcitrance. *Castanea sativa* Mill. is a species widespread in the European continent both in forests and orchards, being appreciated for the nut quality, the wood and the ecosystemic services provided by chestnut groves. This species is currently threatened by two major diseases: ink disease, caused by the oomycete *Phytophthora cinnamomi*, and chestnut blight, induced by the *Cryphonectria parasitica* fungus. New breeding strategies and resources are needed to improve chestnut cultivars in order to plant orchards less susceptible to pathogens. In this paper we report i) the first case of successful application of the CRISPR /Cas9 technology to *Castanea sativa* using the *phytoene desaturase* (*pds*) marker gene and ii) a new technology of protoplasts transfection using ribonucleoproteins (RNPs) targeting the *pds* marker gene. The *pds* gene is involved in chlorophyll biosynthesis and represents a useful marker to test new methods of silencing in plants. In the first experiment (i) somatic embryos of *C. sativa* were modified silencing the *pds* gene using a CRISPR/cas9 construct transferred via *Agrobacterium tumefaciens*. The editing efficiency was about 60%. The CRISPR/Cas9 RNP technology (ii) was then applied to obtain transgene-free plants. Protoplasts were isolated from *C. sativa* somatic embryos using 1.5% cellulase and 0.5% Macerozyme. Results showed

4,500,000 protoplasts/mL with a transfection percentage between 15 and 20%. The technology is currently being used for improving chestnut tolerance to pathogens by silencing the susceptibility genes identified in chestnut in previous research work.

Keywords: *Castanea sativa*, disease, phytoene desaturase, somatic embryogenesis, protoplast, ribonucleoprotein

Selection of new chestnut rootstocks

Author(s):

Ms. Clémence Albert-Talbot, 28 route des Nébouts, 24130 FRANCE Prigonrieux, France; clemence.albert-talbot@ctifl.fr (co-author)
Dr. Fabrice Lheureux, CTIFL, 28, route des nebouts, 24130 Prigonrieux, France; fabrice.lheureux@ctifl.fr (presenting author)

Abstract:

Between 1987 and 1996, a rootstock breeding programme was conducted by INRAE. 6000 hybrids were obtained by controlled pollination (hybrids C. sativa x C. crenata and C. sativa x C. mollissima). An initial evaluation carried out jointly by INRAE, CTIFL and INVENIO made it possible to select 4 rootstocks based on vigour and tolerance to ink disease. We continued this evaluation of these 4 rootstocks, to which were added 2 others from a Portuguese breeding programme 'AMIFEL' ((FB 13 - M 3) and (FD 9 - N 2)), in order to refine, on the one hand, the tolerance to *Phytophthora cinnamomi* and the vigour induced on the Bouche de Bétizac variety, and, on the other hand, to determine their aptitude for propagation, their compatibility for grafting, and their sensitivity to water stress. From this evaluation work, only three rootstocks were retained: (114*599)-20, (75*114)-6 and (577x04)-32, for their resistance to ink disease, the range of induced vigour different from that of the Marsol control, and a lower sensitivity to water stress for the clones (114*599)-20 and (75*114)-6

Keywords: Chestnut, Rootstock, ink disease, vigour, propagation, water stress