GeneWatch UK submission to USDA APHIS docket APHIS-2020-0030: Petition for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut (*Castanea dentata*)[1]

September 2020

This response refers to the **Petition for Determination of Nonregulated status for blight-tolerant Darling 58 American Chestnut**, document number APHIS-2020-0030-0002 (henceforth referred to as the Petition), available on: https://www.regulations.gov/document?D=APHIS-2020-0030-0002

We note that the largest Darling 58 GE trees alive are only about 3 years old, and that no Darling 58 trees are yet mature enough to produce female flowers. This stands in stark contrast to the timescales over which these GE trees will impact ecosystems. Individual trees may live hundreds of years (even if they remain stunted by blight and do not become canopy trees), and the uncontrolled spread of pollen and chestnuts means they will impact ecosystems in perpetuity.

We find that:

- The Petition is seriously premature, due to lack of any long-term data and the many significant gaps in the data that is available.
- Darling 58 GE American Chestnut trees can act as a reservoir for blight, posing a serious risk of infection to native and commercially planted trees, i.e. a plant pest risk.
- The applicants have not established whether the GE trees will show long-term blight tolerance or be able to survive and grow to canopy trees; however, poorly growing (or dying/dead) GE trees will still pose a plant pest risk and may remain part of ecosystems for centuries.
- Numerous long-term ecological risks have not been assessed.
- The applicants have not established whether or not GE pollen will cause allergies, despite the fact this risk may continue in the environment indefinitely if deregulated status is granted.
- There is no precedent for releasing a genetically engineered organism with an antibiotic resistance gene into natural ecosystems, as proposed. This risk has not been addressed at all in the Petition. The use of an antibiotic marker gene in these GE trees poses unnecessary risks and should have been avoided.
- Non-regulated status would make it impossible to trace and monitor the trees, or the GE chestnuts they produce. Distribution of chestnuts and pollen would not be controllable. For example, people could plant GE trees or GE chestnuts in a wide variety of ecosystems, including in countries where such plantings would be illegal. This could occur unwittingly since any chestnut picked up in a forest may turn out to be GE.
- Information provided in the petition regarding unintended pollination, due to late bagging of flowers, seriously undermines confidence in the ability of the applicants to meet permit regulations, as well as highlighting that cross-pollination can easily occur.

[1] https://www.regulations.gov/docket?D=APHIS-2020-0030

- GE chestnuts have yet to be approved as safe to eat, and in any case cannot be labelled if they are not traceable. This has the potential to breach GE food labelling laws and pose a threat to chestnut selling businesses, as well as posing potential risks to human and animal consumers of the chestnuts.
- Deregulation cannot be considered until the GE chestnuts are FDA-approved, as to do otherwise could put human health at risk. Approvals from the Canadian Food Inspection Agency (CFIA) and Health Canada are also necessary prior to considering granting non-regulated status, as GE chestnut tress could be introduced or spread naturally across the border once they are released.
- Even if the FDA does grant approval, it is hard to see how GE food labelling requirements can be met unless the GE trees are traceable. This means they cannot be deregulated.

1. The Petition is seriously premature

The Petition is seriously premature, due to lack of any long-term data and the many significant gaps in the data that is available. This is of particular concern as the planting of the GE chestnuts is likely to lead to the alteration of ecosystems in perpetuity. Deregulation would render the whereabouts of the GE trees unknown and untraceable, and members of the public would no longer know whether chestnuts gathered in a forest, or sold in a market or retailers, were GE or not. Distribution of chestnuts and pollen would not be controllable. For example, people could plant nuts in a wide variety of ecosystems or in countries where such plantings would be illegal. This could occur unwittingly since any chestnut picked up in a forest may turn out to be GE. Once the GE chestnut is released into forests there would be no way to recall it.

The Petition states (p.77) that the largest Darling 58 trees alive at the time of writing are about 3 years old, and (p.144) that, "*No Darling 58 trees are yet mature enough to produce female flowers*…". This means that any long-term properties of these GE trees are completely untested. In addition, very limited testing material is available due to the small number of trees and their limited age (leading to a lack of pollen and chestnuts), and this has affected the statistical power of any studies and/or led to reliance on 'legacy events' which may not give the same results. Finally, many studies have been initiated but not completed (for example, detailed genome analyses), or not done at all with the Darling 58 trees (e.g. studies on tadpole development).

In numerous places, the Petition itself notes that relevant data is not yet available. For example:

- "*Tadpole development and survival*" is highlighted as untested for Darling 58 GE trees (Table 1.3a, p.21).
- "*Additional pollinations with T1 pollen were performed in 2019; inheritance results will be published and/or shared when they are available (testing underway; results anticipated late spring 2020)*". (p.83)
- "*More detailed genome analyses from Darling 58 and offspring will be shared as they become available (anticipated by late 2020)*." (p.88)
- "*A preliminary insert map showing part of Chromosome 7 is shown in Appendix III; further details will be provided when they become available*…" (p.92)

- "*The American chestnut genome is still in draft form and has not yet been annotated, so comparisons to native genes are based on the Chinese chestnut genome…*" (p.92)
- "*According to PCR and limited sequencing data, when Darling 58 sequences are compared to Ellis 1 genomic DNA, Darling 58 has an inversion of approximately 600 base pairs as shown in Figure 7.3.2c, just outside the left border. This inversion is not near any known genes (see above in this subsection). A more complete understanding of the genome sequence near the insertion site should be elucidated by a whole genome sequence of Darling 58 and offspring, which should be available soon as described above*." (p.94)
- The "T1 Nut" samples that have been tested for oxalate oxidase quantities are from transgenic nuts from different mother trees (Figure 7.4.2a, p.100): there are no samples of chestnuts from Darling 58 GE mother trees because there are as yet no female flowers from such trees (as noted on p.144).
- "*We have used several tests to assess blight tolerance on various chestnut tissues and trees, depending on the age and size of material available. This section describes intentional inoculations on Darling 58 tissues and trees using the chestnut blight pathogen Cryphonectria parasitica. Results of inoculations and natural blight infections on older OxO-expressing transgenic chestnut events are described in Section 10.5.1. Further tests on additional outcross generations of Darling 58 offspring will be performed when these trees are large enough to inoculate, and we will continue to share and/or publish results as they become available*." (p.101)
- "*The small number of seedlings available for this inoculation limits statistical power…*" (p.103)
- "*Due to limitations on numbers of available plants, growth rate of tissue culture-generated plants, and the size of field plots, quantitative measurements comparing growth rates and photosynthetic performance of transgenic vs. non-transgenic American chestnut trees have been limited. The most recent available measurements (Section 8.2.2) are from Darling 58 seedling offspring germinated spring 2019; this is the first year a large sample size (> 10 transgenic and non- transgenic seedlings) of Darling 58 seedling offspring has been available for measurement. However, first-season measurements of chestnut seedling height should be considered preliminary as they are not necessarily indicative of future growth, and may be more closely correlated to nut weight, family background, cultural treatments, or other factors*". (p.107)
- "*…data and conclusions should be considered preliminary until measurements can be conducted on older seedlings in controlled experimental plots…*" (p109)
- "*We recognize that these analyses reflect a small number of measurements on a limited number of trees, and that they do not include other non-transgenic American chestnut types that would help put the results in context of natural variation. We also recognize that there could be biological effects of transgene insertion or expression on the photosynthetic and respiratory rates that we were unable to detect here, that such biological effects would only manifest at particular times of year or in particular growth conditions, that any of these effects might be due to linked endogenous chestnut genes near the insertion site rather than the insertion itself, or that such effects may be smaller than those caused by traditional breeding or other treatments. Finally, we have an ongoing effort to more fully characterize the photosynthetic and respiratory physiology of these trees (and others) in three*

common gardens across a climate gradient that will progress over the next few years (see BRAG project description, Section 11.2); results will be published and/or shared as they become available." (p.119)

- "*Real-world exposure of pollinators to OxO depends on transgene expression in pollen, which was not feasible to measure in currently available quantities of transgenic pollen*. *Studies on other transgenic plants suggest that transgene expression controlled by the 35S promoter is negligible in pollen, or expressed at a lower rate than vegetative tissues (see below in this section). Due to limitations on pollen production by transgenic trees, purified barley OxO enzyme (Roche Diagnostics, Mannheim, Germany) was added to non-transgenic chestnut pollen for this experiment*." (p.138)
- "*…many more years of research will be required to produce data about interspecific hybridization of Darling 58 and compatible species*…" (p.144)
- "*Whole genome sequencing is in progress for Darling 4 and the isogenic line WB275-27, which should further clarify details regarding insert location, copy number, structure, etc. Results will be shared when they are available (anticipated in 2020)*". (p.162)
- Long-term research is planned after nonregulated status is granted (p.186)
- Regarding the spread and establishment of chestnut trees, "*Each of these sources has a high degree of uncertainty due to the limited locations or data available, and establishment may be faster on areas with site conditions particularly favorable to chestnut recruitment*". (p.193)
- "*Further sequence analysis of Darling 58 and transgenic offspring is underway; results will be published and/or shared when they become available*". (p. 236)

In addition to these acknowledged limitations, there are many more gaps and omissions, as discussed further below. These relate, for example, to:

- The long-term growth and survival of the trees, and their role in spreading pathogens;
- The long-term dispersal of the trees;
- Implications for human and animal health, including the safety of GE chestnuts for consumption and whether GE pollen will cause allergies;
- Long-term impacts on the complex ecosystems of forests.

Non-regulated status has the potential to lead to a wide range of negative impacts, altering ecosystems in perpetuity. The data supplied in the petition is therefore grossly inadequate to support the application.

2. Creation of plant pest reservoirs in GE American chestnut trees

The applicants state in the Petition that the GE American chestnut trees they wish to plant are tolerant to the fungus *Cryphonectria parasiticia* (Chestnut blight), rather than resistant. The OxO gene in the GE trees produces the enzyme oxalate oxidase, which facilitates the conversion of oxalic acid produced by blight to hydrogen peroxide and carbon dioxide (Petition, p.52). The term tolerance, as used in the Petition (p.64) is a type of plant defence in which the host maintains its fitness or yield despite damage caused by the pathogen. Tolerance still allows the fungus to survive and spread, but aims to limit its damage to the

tree. The applicants emphasise the potential benefit of tolerance, but they do not discuss its downsides. Woodcock et al. (2018) recognise that tolerance may be more durable than resistance, as argued by the applicants (e.g. on p.22 of the Petition): this is because there is less pressure on the fungus to evolve to overcome tolerance than resistance (although this does not mean the applicants can guarantee that tolerance will persist in the longer term, see Section 3). However, Woodcock et al. (2018) also state: "*From a management perspective, tolerant trees do not necessarily negatively affect pest or pathogen populations – such trees could thus become reservoirs for pests and pathogens, with consequences for neighbouring susceptible individuals*".

Based on evidence from the petition, there is little doubt that GE Chestnut trees are expected to act as reservoirs for Chestnut Blight. For example, the Petition notes (p.66), "*In the case of a tolerant host without any toxicity mechanism, all hosts essentially function as refuges*…".The Petition (p.76) states clearly that the blight fungus still colonizes and reproduces on Darling 58 trees, and, "*C. parasitica survives on Darling 58 trees much as it does on Asian chestnuts and other tree species. In fact, Darling 58 remains a suitable food source for the fungus while the tree is alive, and any saprophytic activity of the fungus on dead chestnut tissues would not be affected, since oxalate oxidase production can only occur in living tissues and enzymatic activity stops once tissues dry out*…". The Petition (p. 77) states, "*The life cycle of Cryphonectria parasitica also does not appear to be disrupted by presence or expression of OxO in the American chestnut. We have observed asexual spore reproduction on OxO-expressing trees, and unlike most susceptible chestnuts, cankers on OxO-expressing trees can persist for multiple years after infection without killing the tree (Figure 10.5.1b). Cankers are only reduced in size and severity (Section 8.1), which allows the tree to continue functioning normally*." The Petition confirms the likelihood of GE trees introducing a reservoir of blight on p.147, where it states, "*Dying chestnut trees or dead stems temporarily serve as hosts to the blight fungus while it survives as a saprophyte (Prospero et al., 2006), but ultimately, mortality in a population of entirely disease-susceptible host trees reduces suitable host material for a pathogen. In contrast, Darling 58 can indefinitely serve as a host for the blight fungus. Furthermore, reproductively mature Darling 58 trees will continually produce non-transgenic, blight-susceptible offspring (Section 6.4). Thus it is likely that potential chestnut restoration scenarios including blight-tolerant host trees would not be detrimental to the blight fungus, allowing it to persist by increasing the longevity and/or prevalence of host trees*".

There is no discussion in the Petition of the plant pest risk this inevitably poses to other trees. Introducing GE trees which act as a new reservoir of blight could pose a risk to both native and commercial chestnut trees. Brewer (1995) found that the spread of blight is greater where there are greater numbers of trees that can be infected. Boland et al. (2012) state (p.9) that, "*Locations for new plantings of American Chestnut for restoration or nut crops should be chosen carefully as they may act as a bridge to connect diseased populations of American Chestnut to isolated populations that have escaped disease*". There is therefore a plant pest risk to existing American chestnut, chinquapins, and commercial chestnut trees if GE American chestnut trees are deregulated.

In 2016, around 431 million American chestnut trees remained in the eastern United states (around 10% of the estimated 4 billion before blight, ink disease and logging decimated the

population) (Dalgleish et al., 2016). Dalgleish et al. (2016) estimate that the vast majority of these trees survive as sprouts (an estimated 360 of the 431 million have a diameter of less than 2.5cm at breast height), rather than as large trees. However, some large trees do remain and some are seed bearing. For example, 9 pure American chestnut trees were identified in a competition in New York State in 2016, with the tallest being over 20" in diameter and 80 feet tall: it is not uncommon to be able to collect 100s or 1000s of nuts from such single trees (Fitzsimmons, 2017; Nichols, 2017). In another example, Alexander et al. (2005) report successful location of surviving, seed-bearing, wild American chestnut trees in Tennessee, using a Geographic Information System (GIS): they found 3 trees in 2002, 5 in 2003, and 12 in 2004. Griffin (2000) and Brewer (1995) give other examples of large trees: some are growing in blight-free areas, whilst others are infected with less virulent strains (hypovirulent strains). Since 1986, the American Chestnut Cooperators' Foundation has planted over 135,000 trees of American Chestnut progenies, developed in a breeding programme that promotes low levels of natural resistance, across the native range of American chestnut (Wang et al., 2013). Such trees are examples of the native trees that might be threatened if a new reservoir of blight is introduced by planting blight-tolerant GE trees.

Lovat & Donnelly (2019) rate blight tolerance in the American Chestnut (*C. Dentata*) as Class V (representing the most rapid fungal growth). However, the Allegheny chinquapin (*C. pumila*) and the Ozark chinquapin (*C. ozarkensis*) are also rated as susceptible (Class III or IV), and may therefore also be at risk if the reservoir of blight in native forests is increased. Although the Chinese chestnut (*C. mollissima*) is regarded as tolerant (Class I), chestnut blight appears to be increasingly problematic for commercial plantings, affecting nut production (Lovat & Donnelly, 2019). Thus, chestnut trees grown for commercial nut production in the United States (which are usually hybrids) could also be affected by the presence of new reservoirs of blight on GE trees.

It is important to note that alternative strategies for restoration, and commercial plantings, all involve methods which would limit the spread of blight to some extent: either by making the blight less virulent (hypovirulence), or planting trees that have multiple types of resistance to being infected by blight (e.g. developed through intraspecific or interspecific breeding), and/or through active management measures intended to ensure trees are not infected. For example, there are multiple mechanisms involved in the Chinese chestnut's defence against blight (Petition, p.162). In contrast, the GE trees in the application have only the tolerance trait and are therefore more likely to act as reservoirs of blight, with potential negative implications for other trees. The planting of GE trees likely to become infected with blight also has the potential to undermine some alternative control strategies: for example, those based on reducing the virulence of blight (hypovirulence) or on using sanitation measures (which rely on removing infection sites and infested material, and preventing the spread of infected material), or on planting low-blight-resistant native bred trees.

Brewer (1995) describes trees in Michigan which are recovering from blight, through the development of less virulent strains (hypovirulence), 15-25 years after blight infects an area. The impact of planting GE blight-tolerant trees on the development and spread of hypovirulent strains, which can allow wild-type American chestnut trees to recover, is also not discussed in the Petition. It is possible that planting blight-tolerant GE trees would allow

virulent forms of chestnut blight to grow and spread more easily and thus prevent recovery of wild-type trees from blight.

This potential role of GE blight-tolerant trees as reservoirs of infection means they post a plant pest risk and therefore should not be deregulated under the Plant Protection Act.

3. Inadequate timescales to evaluate blight-tolerance

Not only do the proposed GE trees post a pest risk by acting as reservoirs of blight, their long-term tolerance to blight has not been established. This is relevant because trees that do not survive to fulfil the desired role as canopy trees can do more harm than good by acting as reservoirs of blight, as discussed in Section 2, without providing the claimed benefits.

The Petition states (p.77) that the largest Darling 58 trees alive at the time of writing are about 3 years old. These are very young trees that are below the usual age of maturity and younger than the age at which American Chestnuts usually succumb to blight, let alone the age they would need to reach to fulfil the claimed beneficial role as canopy trees in forests.

Time is needed to be confident in the resistance/tolerance of trees to blight and their viability (Woodcock et al., 2018). Clark et al. (2014) note in relation to trials of conventionally-bred blight-resistant trees that, "*These tests were short-term evaluations of very young trees (<4 years old) in a relatively uniform environment*", and that juvenile resistance may be affecting results. Fitzsimmons (2017) reports that, "*Most American chestnut orchards become overwhelmed by blight by year 5 and, by year 10, virtually all original stems will be eliminated*". In addition, she notes that most trees in such orchards will produce chestnuts for harvest before they succumb, following which most of the trees will resprout, and about 50% will survive for decades as resprouts. This article includes a photograph of a ten-year-old, 30ft American Chestnut tree in a Germplasm Conservation Orchard (GCO), and surrounding similar trees in the orchard. This ten-year timescale is notably longer than the 3-year age of the oldest Darling 58 GE trees.

The Petition notes (p.31) that American chestnut trees live to an age of 400 to 600 years, though trees over 100 years grow hollow in the centre, and that it takes approximately 10 to 15 years for American Chestnut trees to reach 10m height under optimal conditions. According to the Petition (p.33) American chestnuts can produce seed at 8 to 10 years, but regular and plentiful crops appear only after 20 years, and some trees in natural conditions may not produce seed until 20 years. The Petition also states that American chestnuts in orchards may begin producing seed at an age of 4 to 6 years, although production of female burs generally lags behind production of male catkins by about 2 years (Petition, p.42). The T1 generation of trees (GE trees crossed with wild American chestnuts) were produced by accelerating pollen production to less than one year using light growth chambers (Petition, p.42). Thus, the 3-year age of the oldest Darling 58 GE trees is considerably shorter than the expected lifespan of an American chestnut tree or the time needed for such a tree to grow sufficiently to become a canopy tree, or even to produce female flowers and chestnuts.

There is no way to be confident that the GE blight-tolerant trees will remain blight-tolerant throughout the normal adult lifespan of an American Chestnut tree, except by conducting much longer-term experiments. Although oxalate (oxalic acid, OA) is a confirmed virulence factor for blight, there are many other likely or suspected virulence factors (Lovat & Donnelly, 2019). It is possible that these alter the trees response to blight over longer timescales. It also possible that chestnut blight (*C. parasit*ica) evolves to produce more oxalic acid once it has infected GE trees, or other developments take place that reduce the blight-tolerance effect.

In addition, the applicants have chosen not to wait for the results of further tests on additional outcross generations of Darling 58 GE trees (Petition, p.58). Given that they envisage a multi-generational product involving the gradual spread of GE trees into the wild, the limited number of generations tested is also inadequate. Further, although leaf inoculations can play some role in showing proof of principle (p.101-102), the number of stem innoculations performed has been extremely limited: 12 small stems were inoculated in greenhouses in 2016, of which only 9 are still healthy (p.103); 3 field-growing trees of each transgenic Darling 58 offspring in the T1 generation (p.103) were inoculated in late summer of 2018, which the applicants admit have limited statistical power; and 58 potted Darling 58 T1 seedlings were innoculated. According to the Petition (p.103), all 3 field-growing inoculated GE trees had swelling cankers that did not girdle the trees by the end of the 2018 growing season (in comparison with the non-transgenic controls which were girdled): however, no information is provided about longer-term survival. In the experiments using potted trees, 20 stems were removed from the analysis due either to not developing visible signs of infection (18 stems across all types), or due to dying (2 stems across all types), leaving 36 GE potted trees, compared to 24 controls (Figure 8.1.3b, p.105). Whilst results show slower canker progression and smaller canker heights (with lack of girdling) in the GE trees compared to the controls, these experiments continued for only 29 days after inoculation.

The Petition states (p.147), "*We do not yet have experimental data on infection and reproduction rates of the chestnut blight fungus on large Darling 58 trees in a forest setting, but a long-term experiment has been initiated that will provide data on these questions in the years to come*…". It is clearly premature to consider deregulation of these GE trees in the absence of such data.

The lack of demonstration of blight-tolerance over much longer timescales, relevant to the survival to adulthood, nut production, and full growth, is relevant because trees that do not survive to fulfil the desired role as canopy trees can do more harm than good by acting as reservoirs of blight, as discussed in Section 2.

4. Durability of GE American Chestnut trees is not established

Due to the short timescale of existence of the Darling 58 GE trees (up to 3 years), as discussed above, the broader durability of these trees (apart from their blight tolerance characteristics) has also not been adequately assessed. This is relevant because trees that do not survive to fulfil the desired role as canopy trees can do more harm than good by acting as reservoirs of blight, as discussed in Section 2. It is important to note that even

poorly growing (or dying/dead) GE trees will still pose a plant pest risk and may remain part of ecosystems for centuries, but poorly growing trees will not become canopy trees and thus not deliver the desired outcomes.

The Petition (p. 4) notes that some of the second-generation (T2) offspring appear to show slower first-season growth of transgenic compared to non-transgenic seedlings: however, long-term growth characteristics are unknown. This is because of the short time scale over which these trees have been studied (see Section 3), which is totally inadequate to demonstrate their long-term durability.

The petition (p.106) refers to over 13 years of anecdotal unpublished observations: however, these mostly relate to different versions of the GE trees (referred to as legacy trees in the Petition), and in any case this is still short compared to relevant timescales discussed in Section 3. The Petition admits (p.107) that the numbers of GE trees available to study have been limited and that Darling 58 seedling offspring germinated in spring 2019 provide the first sample size of more than 10 transgenic and non-transgenic seedlings. The Petition (p.109-111) downplays some evidence of reduction in growth rates, as being based on small numbers and perhaps not representative of long-term rates, and argues that better long-term growth data will be obtained in future. Whilst this might be the case, the opposite might also be the case (and is probably more likely). The data provided is clearly insufficient to establish that long-term growth will be adequate to fulfil the claimed objectives of producing fully grown blight-tolerant canopy trees.

The Petition also recognises (p.119) that studies of photosynthesis and respiration "*reflect a small number of measurements on a limited number of trees*".

There are numerous reasons why growth of these GE trees might be poor, such as the metabolic cost of expressing the OxO enzyme (mentioned on p.110 and p.116-117 of the Petition), or other effects related to the impacts of the genetic changes on the metabolism of the trees, or their interactions with the environment (including blight infections). For example, the main by-products of degradation of oxalic acid by OxO are hydrogen peroxide and carbon dioxide and the Petition argues (p.57) that continuous moderate concentrations of hydrogen peroxide are not generally harmful to plants. However, it also notes (p.58) that sometimes hydrogen peroxide can also exacerbate damage, as described by Van Breusegem et al. (2001). The existence of such complexities highlights the importance of studying the growth of blight-tolerant GE trees over much longer timescales before deregulation can be contemplated.

The broader resilience of the trees in their native habitats is also completely unknown, and there are many reasons to expect this would be poor. Westbrook et al. (2020) notes that the GE trees, established from a single clone, will have very limited diversity, and proposes a theoretical plan for diversifying the GE tree population by cross-breeding with wild-type American chestnuts. As well as carrying potentially significant environmental risks (see Section 5), there is no data in the Petition to determine whether or not such trees would be robust. Further, the plan outlined by Westbrook et al. (2020) also relies on the development of different transgenic founders expressing OxO using different promoters, in order to attempt to reduce the risk of gene silencing (leading to trees which succumb to blight). This

is also an untested strategy, which presumes that future GE trees will be successfully developed and further petitions for deregulated status will be granted.

One concern is the resilience of the trees in the context of multiple stressors. Woodcock et al. (2018) note that, "*trees that are resistant to a specific pest or pathogen do not necessarily withstand other pressures experienced in the field (e.g. climate stress, other pests and pathogens, herbivory etc.)*" and that these additional pressures affect the success of resistant tree programmes, and can compromise resistance mechanisms. In relation to experiments with conventionally-bred blight-resistant trees, they note that, "*whilst blight-resistant American chestnut experienced low impacts from the target pathogen during field trials, mortality rates nonetheless ranged from 12 to 70 per cent, primarily due to Phytophthora cinnamomi Rands, as well as deer browsing and insect damage (Clark et al., 2014)". Phytophthora cinnamomi* Rands cause ink disease, which first arrived in the USA in the early 19th century and caused widespread mortality in the southern portion of the American Chestnut species (Clark et al., 2014). Clark et al. (2014) note that blight incidence was less than 10% in all the tree plantings discussed in their paper, and mortality was primarily related to the ink disease pathogen. The Petition (p.143) reports some experiments in which nearly all (transgenic and non-transgenic) seedlings succumbed to *Phytophthora*. This suggests that the GE trees are unlikely to survive infection with ink disease. It should also be noted that the cited study by McKeever et al. (2019) appears to be unavailable for public scrutiny.

Other factors noted by Clark et al. (2014) (which were significant in some specific trials), included a root rot disease (caused by a *Phthium* species or a severe drought) and invasive exotic insects, including the Asian gall wasp and the Asiatic oak weevil. Animal damage is caused by deer (to seedlings) and consumption of nuts by predators, including rodents. Gypsy moth also feeds on American Chestnut, although it did not affect these particular trials. Clark et al. (2014) further note that limitations in cold tolerance could predispose trees in the northern range and at high elevations to disease pressure from blight, and these authors highlight the importance of assessing gene-environment interactions.

In summary, Clark et al. (2014) state that, "*Current restoration efforts have primarily focused on production of trees resistant to one of these pests, the chestnut blight (Cryphonectria parasitica), but landscape-level restoration will require much more than a blight-resistant tree for deployment*." They note that, "*Today restoration is more difficult than in any previous time, because the American chestnut continues to be plagued by multiple exotic and native pests*", and caution that, "*We must guard against rushed attempts and overly optimistic expectations that could lead to significant ecological damage and a public that loses faith in the effort*".

In general, low genetic variation can increase susceptibility to environmental stressors and make tree populations less durable in the longer term (Woodcock et al., 2018). Thus, any attempt to create a durable population of trees from a single clone is fraught with extreme difficulties.

The durability of the GE trees is relevant to the issue of plant pest risk because poorly surviving GE trees can nevertheless act as a reservoir of blight, as discussed in Section 2.

Short-lived or poorly growing GE trees could act as reservoirs for blight, presenting a plant pest risk to other trees, without providing any benefits (such as the claimed replacement of canopy trees over the longer term).

5. Inadequate studies and regulatory oversight regarding impacts on the environment and wildlife

The Petition states (p. 23) that "*Oxalate oxidase (OxO) is not intended to prevent, destroy, repel, or mitigate the blight fungus, so it is different from products subject to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA)*" and that "*the precise role of the EPA in reviewing this unique product is still being discussed*", although the Petition will be shared with the EPA. Whether or not a registration is required under FIFRA therefore appears to be a matter of dispute. It should be noted that, in granting a recent experimental permit under FIFRA for genetically engineered mosquitoes, the EPA stated that issuing a permit under FIFRA would mean that the EPA is not required to conduct a full Environmental Impact Study (EIS) under the National Environmental Policy Act (NEPA) (p.137-139, EPA Response to Comments[2]). If this analysis is correct, it would imply that a full analysis under NEPA is required in circumstances when an application under FIFRA is not made. In addition, there are relevant obligations under the Endangered Species Act (ESA).

Since these are not necessarily matters for the USDA APHIS, we do not discuss the environmental impacts in detail below. However, we note that the information provided in the Petition is inadequate to meet the necessary legal requirements of the NEPA and the ESA. Further, it is premature to deregulate in the absence of an EIS, as deregulated trees will not be traceable (see Section 6).

In a survey, Russin et al. (1984) captured 495 insect species feeding on American chestnut stems and chestnut blight cankers. Lovat & Donnelly (2019) describe numerous microbial species that might either help or hinder an ongoing pathogenic infection, including some known to inhibit chestnut blight growth. These studies merely hint at the complexity of the ecology of the American Chestnut and its numerous interactions with other species and the wider environment. As noted in the Petition, chestnuts also provide a nut crop that is consumed by numerous mammals, birds and insects (p.18, p.33, p.36) and multiple bee species and other pollen feeders (e.g. beetles and moths) have been observed visiting catkins (p. 32, p.37). There is evidence that that leaf shredding insect species prefer chestnut leaf litter to that from other tree species, and that mayfly larvae also prefer American chestnut leaves (Petition, p.37). Chestnut leaf litter also provides nutrition for aquatic macroinvertebrates (Petition, p.42). In this context, the studies presented in the application are extremely limited, and inadequate to demonstrate that GE American Chestnut trees will not impact negatively on the environment and wildlife.

Relevant studies provided in the Petition (Table 1.3a, p.21) are limited to:

- Nutritional analysis (compared to non-GE) (Section 8.4.1, and Section 10.5.2 for Darling 4)

[2] Response to Comments OX5034 to the Notice of Receipt of an Application for an Experimental Use Permit Number 93167-EUP-E. ID: EPA-HQ-OPP-2019-0274-0355. https://beta.regulations.gov/document/EPA-HQ-OPP-2019-0274-0355

- Mycorrhizal fungi (compared to non-GE) (Section 9.1.1, and Sections 9.1.1 and 10.6.1.2 for legacy events).
- Native plant interactions (Section 9.1.2, and Section 10.6.5 for legacy events)
- Insect herbivory (Section 9.1.3, and Section 10.66-10.7 for legacy events)
- Bumble bee use of pollen (Section 9.1.4)
- Response to other plants (Section 9.2, and Section 10.6.9 for legacy events)
- Transgenic leaf litter: decomposition, OxO persistence (Section 9.4, and Section 10.6.3-10.6.4 for legacy events)
- Tadpole development and survival (compared to non-GE) (Section 10.6.8 for legacy events only)

These tests consider only a tiny proportion of relevant species and potential mechanisms for harm. In addition, legacy events are earlier versions of the Darling 58 GE trees, which may have different properties. For example, it is completely unacceptable that only legacy events have been studied in relation to tadpole development and survival, since they may not give a true indication of the risks posed by Darling 58 GE trees.

In general, there is an over-reliance on 'bridging data' from legacy events, and a tendency to dismiss adverse outcomes when they do occur (Section 10). Where risks in legacy events have been noted, they have not been taken seriously. For example, the petition notes (p. 178), "*For gypsy moths, growth of larvae fed on Wirsig leaves was significantly faster (16%; $p < 0.012$) than growth of larvae fed on wild-type leaves. If gypsy moth growth is consistently faster on OxO- producing chestnuts than non-transgenic chestnuts, this could potentially indicate a novel pest risk by enhancing gypsy moth outbreaks*". This risk is then dismissed as "*not likely applicable to Darling 58*", instead of prompting further studies. Differences in fungal species diversity were also noted, then dismissed, instead of prompting more in-depth investigation (p.174), "*The analysis found fungal species diversity to be higher in Zoar than in Darling 4. Diversity in Hinchee 1 leaf litter was lower than Zoar and Darling 4 (Table 10.6.3a). The largest difference, that between Hinchee 1 and Zoar, may be caused by the antimicrobial peptide gene present in the Hinchee events (See Section 10.1; because of this, Hinchee 1 is not intended to be used for bridging data). Alternatively, this difference, and the smaller difference between Zoar and Darling 4, may be an artifact of low sample size. Only 10 litterbags were deployed for each leaf type, and rarefaction curves showed the number of OTUs continuing to increase for all leaf types, indicating that the study did not fully capture the diversity of fungal OTUs that would ultimately colonize leaf litter of the three leaf types. Because of this, Gray warns that despite statistical significance, inferences about differences in diversity are "most likely premature," and that "overall, the process of genetic engineering using the transgene oxalate oxidase does not appear to have any measurable effect on the diversity of fungi that colonize leaf litter*."

The Petition (p.86) notes that the OxO gene in Darling 58 GE trees is driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter and that "*expression under this promoter is considered to be high and present in nearly all tissues, with the possible exceptions of dry seeds, pre-germinated embryos, and pollen, in which expression can be lower or negligible*". The promoter in Darling 58 expresses more OxO in leaves than stems, and more in stems than in nuts and roots, however expression was more than an order of magnitude higher than in food grain sources (Petition, p.96 and p.99-100). OxO has not yet

been quantified in pollen from the Darling 58 GE trees (Petition, p.127), which is another serious omission from the data. The "T1 Nut" samples that have been tested for oxalate oxidase quantities are from transgenic nuts from different mother trees (Figure 7.4.2a, p.100): there are no samples of chestnuts from Darling 58 GE mother trees because there are as yet no female flowers from such trees (as noted on p.144). This is yet another major omission from the data.

The impacts of OxO expression throughout the tree, throughout its lifetime, remain essentially unknown.

Impacts on other species can occur through direct, indirect or complex mechanisms. The main by-products of degradation of oxalic acid by OxO are hydrogen peroxide and carbon dioxide. Ramputh et al. (2002) describe GE corn transformed with a wheat oxalate oxidase (OXO) gene and its impacts on the European corn borer (*Ostrinia nubilalis*). Tunnelling of the corn borer was reduced by 50%, possibly due to lignification of the plant's cell walls (due to the presence of additional hydrogen peroxide), or due to direct or indirect negative effects of hydrogen peroxide on the corn borer. Mao et al. (2007) study a similar GE corn and find a significant increase in ferulic acid, which reduced larval growth rates. Whilst in these papers the authors report intended negative impacts on a pest, these studies should also raise concerns that expression of OxO in GE Chestnut trees, and the associated production of hydrogen peroxide or other products, could have unintended negative impacts on other (non-pest) species.

The Petition suggests that hydrogen peroxide will only be produced around cankers, as it is produced by the degradation of oxalic acid produced by the chestnut blight. However, numerous other fungi are likely to occur in association with chestnut trees, particularly in soils. In other transgenic plants, OxO genes have been used to seek to protect poplar trees from *Septoria musiva* (leaf spots and canker disease) and potatoes against *Phytophthora infestans* (late blight) (Ilyas et al., 2016) and taro against *Phytophthora colocasiae* (taro leaf blight) (Moosa et al., 2017). This suggests there is at least a possibility that hydrogen peroxide will be produced on roots and leaves of GE chestnut trees in reaction to other pathogens. The environmental consequences are unknown.

The applicants have not established whether or not GE pollen will cause allergies, despite the fact this risk may continue in the environment indefinitely if deregulated status is granted. For example, transgene expression in pollen *"was not feasible to measure in currently available quantities of transgenic pollen"* (p.138) and "*OxO has not yet been quantified in Darling 58 pollen…*" (p. 127).

The extent to which OxO expression changes other properties of the GE trees is also unknown. For example, Section 2.2.3 of the Petition (p.34 to 35) discusses American chestnut and fire. However, there is no consideration of whether the production of hydrogen peroxide will make the trees more flammable. Hydrogen peroxide is not combustible but it is a strong oxidiser which enhances the combustion of other substances.

In general, the data provided is completely inadequate to assess the impacts of the GE trees on ecosystems. Once deregulated and released into forests, such impacts could last in perpetuity.

6. Use of an antibiotic resistance marker gene

The petition states (p.8), that, in addition to the gene for OxO, a selectable marker called neomycin phosphotransferase (NPTII) was added for use in the development of the GE trees. Expression of NPTII (from *Escherichia coli*) allows plant tissue to survive in the presence of aminoglycoside antibiotics such as kanamycin; neomycin; geneticin (G418), or paromomycin: the latter served as a selectable marker during the development of the GE trees, facilitating development and selection of transformed lines. This marker is discussed very briefly in Section 7.1.2 of the Petition (p.87), where the applicants state that the NPTII gene does not have any reported plant pest characteristics and has been used in several plants previously deregulated by USDA-APHIS.

The Petition states (p.144) that, "*The only logical means by which transgenes from Darling 58 could spread to related species is through inheritance by viable offspring from successful pollination with at least one transgenic parent*". However, the Petition later notes (p. 145), "*Horizontal gene transfer, which can occur if microbes incorporate plant genes into their own genomes, is also theoretically possible*" (p.145). Horizontal gene transfer (HGT) is mentioned only in the context of the OxO gene. However, it is arguably more important to consider in the context of the antibiotic-resistant gene (ARG) used as a marker in these GE trees.

Historically, several lines of transgenic or genetically engineered (GE) crops have incorporated one or more antibiotic resistance genes (ARGs) including neomycin (Gardner et al., 2019). However, concerns have long existed that horizontal gene transfer could lead to the transfer of antibiotic resistance to micro-organisms such as bacteria, contributing to the growing global problem of antibiotic resistance. Chen et al. (2012) detected antibiotic resistance genes from synthetic genes used in genetic engineering in Chinese rivers: resistance genes sourced from synthetic plasmids released into the environment may therefore be a source of antibiotic resistance. Recently, Gardner et al. (2019) have published evidence that that crop-derived transgenes contained within digested transgenic foods may enter wastewater treatment plants. These authors detected significant levels of nptII in extracellular DNA in batch reactor experiments after 60 days and warned that the use of ARGs in transgenic crops could lead to a persistent reservoir of ARGs in waste water systems.

In the case of GE trees, the ARGs incorporated into the tree are presumably expressed throughout the tree and could enter the environment and the food chain in multiple ways. Given the extremely long timescales involved in populating forests with full-grown GE American chestnut trees, the use of an ARG is a matter of significant concern. There is no precedent for releasing a genetically engineered organism with an antibiotic resistance gene into natural ecosystems, as proposed in the Petition. This risk has not been addressed at all in the Petition.

The use of an antibiotic marker gene poses unnecessary risks and should have been avoided.

7. Cross-pollination, traceability, monitoring, food labelling and irreversibility

Deregulation would severely limit the potential to monitor the GE trees, because individual trees (and their offspring) will not be traceable. This is a serious issue in the context of the need for monitoring of tree resistance and environmental impacts, but traceability is also essential in order to implement food labelling laws. An important consequence of lack of traceability is the inability to intervene to remove trees or mitigate damage, should problems arise. Traceability is also essential to prevent food contamination incidents, in which GE chestnuts inadvertently end up in commercial products which are required to be non-GE (e.g. organic products or exports), or to be labelled as GE.

Monitoring is essential in any programme involving the planting of resistant/tolerant trees, at minimum to: detect cases in which tree resistance is being overcome; evaluate survival and causes of mortality; inform breeding programmes and strategies to maintain resistant trees (Woodcock et al., 2018). However, it is also necessary for environmental reasons, and to protect the food chain.

The applicants intend to make deregulated America Chestnut trees widely available to members of the public and 'citizen scientists' (Petition, p.186; Westbrook et al., 2020). The focus is on outcrossing to increase genetic diversity, using a combination of pollen, scion wood for grafting, seed and seedlings, to distribute the GE trees (Petition, p.42). However, citizen science records are often opportunistic and stochastic and cannot inform on all regions of interest (Woodcock et al., 2018). People will be encouraged to deliberately cross-pollinate the GE trees with wild-type trees (Petition, p.186). This is in essence a plan to make the distribution of GE trees untraceable and hence unmonitorable in any meaningful sense.

The Petition states (p. 33) that chestnut pollen can travel up to 100km, but that effective pollination distances are much shorter and trees further than 300 to 400m apart will generally not pollinate each other. However, even if correct, this will not limit the spread of the trees if people are being encouraged to deliberately cross-pollinate them. The speed of spread is not the key issue here, it is the fact that any spread would be irreversible and the GE trees would not be traceable. Distribution of chestnuts and pollen would not be controllable. For example, people could plant GE trees or GE chestnuts in a wide variety of ecosystems, including in countries where such plantings would be illegal. This could occur unwittingly since any chestnut picked up in a forest may turn out to be GE.

There also implications for native chinquapins and for commercial chestnut trees (including European and Asian chestnuts), as the GE chestnut trees may hybridise with them. The Petition notes (p. 24) that species in the chestnut genus (which include the Ozark chinquapin, Allegheny chinquapin and European and Asian chestnut species) have the potential to hybridize in zones where two or more species overlap. The chinquapins occupy much of their pre-blight range, though in reduced numbers, and the North American Castanea species appear to hybridize where their distributions overlap (Petition, p. 26).

Most other (European or Asian) species of *Castanea* can also freely hybridize with American chestnut (Petition, p.29). Although there is only one report of naturalized Asian or hybrid chestnut populations In North America, Asian and hybrid chestnut species are grown commercially and some pure American chestnuts are also grown commercially to meet the demand for nuts from native species (Petition, p. 30). The Petition also notes (p. 42) that American chestnut can hybridize with native chinquapins and that hybrids with the GE trait of blight tolerance could potentially have a competitive advantage over blight-susceptible chinquapins. However, the Petition does not discuss what might happen to chinquapins in the longer term if blight-tolerance is only temporary or if such hybrids do not survive in the longer term for other reasons (See Sections 3 and 4 of this response). As noted in Section 2, the role of GE chestnuts as a reservoir of blight may also pose a threat to chinquapins and to non-native chestnut trees.

The Petition (p. 58) refers to controlled pollinations with *C. dentata x C. mollissima* F1 hybrids, Allegheny chinquapin, and European chestnut (unpublished data) and states that these offspring generally appear healthy but are not yet of flowering age, so there is no data regarding rates of male sterility or seed production in subsequent generations. The Petition (p.58) notes that "*many more years of research will be required to produce data about interspecific hybridization of Darling 58 and compatible species*" but goes on to speculate that natural hybridization events would be rare. This is yet another example of the lack of data, illustrating that the Petition is seriously premature.

Moreover, the Petition itself highlights how unintended cross-pollination could occur. The Petition states (p. 82) that apparently low inheritance rates of the OxO gene in field trials in the 2016 pollination season, "*may be explained by late pre-bagging of female flowers (which could have allowed pollen from other non-transgenic trees to have pollinated the flowers before bagging)*…". The caption to Table 6.4a (p. 83) also states that, "*Field records indicate starred mother trees were pre-bagged after some female flowers may have ripened, possibly resulting in pollination by airborne non-transgenic pollen*". As well as the 2016 cross-pollinations, 6 of the cross-pollinations in 2018 are also starred. Further it appears to contradict p.144 of the Petition, which states that, "*No Darling 58 trees are yet mature enough to produce female flowers, and any male flowers have been either bagged or produced indoors to comply with APHIS permit regulations, so we have no data on natural pollination rates for Darling 58 trees*". Earlier problems with late bagging of the legacy Darling 5 GE trees (between 2012 and 2015) are described on p.152 of the Petition, which states that the observed less than 50% inheritance rate "*likely reflects unintended open pollination due to inefficient pre-bagging of female flowers before controlled pollination*". This information seriously undermines confidence in the ability of the applicants to meet permit regulations, as well as highlighting that unintended pollination can easily occur.

The lack of traceability also has major implications for the food chain. Clark et al. (2014) note that, in the past, Native Americans valued the American Chestnut tree as a high-quality food source and used the tree for medicinal reasons. The nuts were enjoyed raw, roasted or boiled by Native Americans and settlers (Wang et al., 2013). Chestnut trees are now grown for commercial nut production in the United States. Some producers use hybrid chestnut trees (such as 'Colossal', a Japanese-European hybrid) (Anagnostakis, 2012). Chestnut growers are represented by the Chestnut Growers of America

(http://www.chestnutgrowers.org/) and the Michigan Chestnut Cooperative (www.chestnutgrowersinc.com). Markets for such chestnuts may be threatened by the risk of cross-contamination by GE trees and lack of traceability of GE trees may also make it impossible to implement labelling requirements.

The Petition (p. 23) notes that nuts from the GE trees "*will likely be consumed by humans and livestock*" and states that, in addition to the USDA, documentation on the Darling 58 blight-tolerant American chestnut trees will be submitted to the FDA for review. The applicants "*do not anticipate pure American chestnuts becoming a prominent agricultural product*" but state that "*if, or when, healthy American chestnuts are able to mature, flower, and produce nuts in the wild, it is almost certain that the nuts will be readily consumed by both people and wildlife*" (Petition, p. 119-120). However, claims regarding the limited status of chestnuts as an agricultural product are highly speculative given the long timescales involved. For example, Wang et al. (2013) note that during the late 19th century and early 20th century, chestnuts were a popular treat in large cities and could be a profitable small business venture.

Deregulation by USDA APHIS would mean the GE trees are not traceable. If the GE trees are not traceable, it is difficult to see how legally required labelling requirements will be met.

The National Bioengineered Food Disclosure Standard has been adopted as a requirement of the National Bioengineered Food Disclosure Law, passed by Congress in 2016.[3] The implementation date of the Standard is January 1, 2020, except for small food manufacturers, whose implementation date is January 1, 2021. The mandatory compliance date is January 1, 2022. The Standard requires food manufacturers, importers, and certain retailers to ensure bioengineered (BE) foods are appropriately disclosed. The Standard defines bioengineered foods as those that contain detectable genetic material that has been modified through certain lab techniques and cannot be created through conventional breeding or found in nature. Chestnuts from the GE Chestnut trees considered in this consultation appear to meet the requirements to require a bioengineered food disclosure. The Standard requires regulated entities, which includes food manufacturers, importers and certain retailers, to ensure bioengineered foods are appropriately disclosed. Retailers who package food or sell food in a bulk container and/or display are responsible for ensuring that any bioengineered food bears a BE disclosure or that a bulk display includes signage identifying the food as BE. However, 'very small' food manufacturers (with annual receipts below $2,500,000) are exempt, as is food served in restaurants or similar retail food establishments.

In addition to meeting the National Bioengineered Food Disclosure Standard requirements, some producers will need to demonstrate that their chestnuts are non-GE, in order to meet organic food standards, and exports will be impossible unless regulatory approvals are obtained in other countries (many of which will also require labelling).

A detailed discussion of food safety issues is not attempted here, as this is likely a matter for the FDA. However, we note that, in comparisons with food grain sources, the Petition states

[3] https://www.ams.usda.gov/rules-regulations/be

(p.99), "*All food grain sources showed more than an order of magnitude less OxO than the transgenic chestnut tissues*…" (see also Figure 7.4.2a on p.100) and accepts that "*OxO consumption in a single serving of transgenic chestnuts (i.e. acute exposure) would likely be higher than that from a single serving of wheat or other foods, given the relatively higher expression of OxO in Darling 58 chestnuts*" (p.127). Due to this substantial difference, the Petition looks to medical applications for comparisons: however, it is not clear that such standards are sufficient to protect people consuming the nuts and no feeding studies of the chestnuts to rodents are reported. It should be noted that, unlike other OxO sources, such as wheat, chestnuts are predominately consumed unprocessed (raw or cooked for human consumption), although chestnut flour is also produced. Again, the information provided is inadequate and therefore the Petition is premature. For example, full genome sequencing of the GE trees is underway, however more detailed analyses are not yet available (Petition, p.87-88). Figure 7.4.2a (p.100) notes that the "T1 Nut" samples that have been tested are from transgenic nuts from different mother trees: there are no samples of chestnuts from Darling 58 GE mother trees because there are as yet no female flowers (p.144). Thus, it is premature to assume that the FDA would automatically allow GE chestnuts on the market: deregulation cannot therefore be considered until the GE chestnuts are FDA-approved, as to do otherwise could put human health at risk. Even if the FDA does grant approval, it is hard to see how labelling requirements can be met unless the GE trees are traceable. This means they cannot be deregulated.

The Petition notes that regulatory submissions are anticipated to the Canadian Food Inspection Agency (CFIA) and Health Canada in the future (p. 23). It states (p.230, "*The natural range of the American chestnut extends into Canada, so Darling 58 trees may be introduced or eventually naturally introgress across the border*". However, because deregulation would lead to lack of traceability of GE trees and GE chestnuts (as noted above), it is (again, at best) premature, until these Canadian regulatory approvals have also been granted.

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