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Drivers of Forest Pathogen Invasions: The Roles of Global Trade and Climate Change

Steve Woodward[1], Refika Ceyda Beram[2], Hatice Tuğba Doğmuş[3]*

(1) University of Aberdeen, School of Biological Sciences, Plant and Soil Science, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3DU, Scotland, UK; (2) Pamukkale University, Faculty of Arts and Sciences, 20160, Denizli, Turkey; (3) Isparta University of Applied Sciences, Faculty of Forestry, 32260, Isparta, Turkey

* Correspondence: e-mail: tugbadogmus@isparta.edu.tr

ABSTRACT

In the past 25 to 30 years, a marked increase has occurred in numbers of invasive alien pests and pathogens recorded, damaging agriculture, horticulture and forest environments. The reasons for this upsurge are clear: all involve human-driven factors, with, arguably, global trade as the most important component in the complex. Climate change is another major factor. Each plant transported through trade could carry cryptic pests or pathogens, which may be introduced into previously naïve environments and lead to major health issues on previously unaffected plants. Global trade in plants is a complex system, driven by a desire to use large plants in landscapes and demand for varied species for gardens. Examples of invasive alien pathogens causing wide-spread problems on a global scale currently include many *Phytophthora* species, Dothistroma needle blight of pines, and *Xylella fastidiosa* on a wide range of trees. A striking example recently was the spread of the Dutch elm disease pathogen, *Ophiostoma novo-ulmi*, in North America and Western Europe. Many invasive pathogens cause problems in localized regions, including *Ceratocystis platani*, killing *Platanus* in some European countries. Other examples include the threat of pine wilt nematode (*Bursaphelenchus xylophilus*), damage to *Cupressus* in Mediterranean landscapes caused by *Seiridium cardinale*, dieback of sweet chestnut (*Castanea*) caused by the canker pathogen *Cryphonectria parasitica*, and many others. In addition to global trade, climate change appears to be exacerbating the problems, with major influences on establishment and spread of alien invasive species. Hope lies in the enormous genetic 'power' of plants: humans could establish selection and breeding programmes that will ultimately enable us to plant trees with greater tolerance of changing climate and pests and pathogens, whether native or invasive.

Keywords: Invasive alien pathogens; Global trade; *Phytophthora*; *Ophiostoma*; *Dothistroma*; *Ceratocystis*; *Cryphonectria*

INTRODUCTION

In the past 25 to 30 years, there has been a marked increase in the numbers of alien invasive pests and pathogens of plants recorded in populated continents of earth, damaging agriculture, horticulture and the wider environment, including both natural and plantation forest ecosystems. The reasons for this upsurge are clear: all involve interacting human-driven factors, with, arguably, global trade as the most important component in the complex (DAISIE 2014, Brockerhoff and Liebhold 2017, Santini et al. 2018). Climate change also appears to be having an impact on the establishment and impact of invasive pests and pathogens (Jactel et al. 2020).

This review focuses on increased damage in forest ecosystems by invasive alien species (IAS), with focus on invasive pathogens, some of which have led to, or are threatening, near extinction events for several tree genera and species. This review is focused on some of the most damaging and significant IAS of pathogens that are present in European forests.

The History of Global Spread of Damaging Forest Pathogens

Forests are ecosystems of enormous biological productivity, a fraction of which is utilized by humans in commerce, in timber production, for example, or in non-timber forest products. It is a fact, however, that in all of

these ecosystems, some productivity is lost to biotic agents and abiotic factors associated with the forests. The forests in Figure 1, for example, all look pristine, with no pests or diseases apparent. Despite the appearance, however, each tree in each image will be colonised in to some extent by fungi and bacteria which may, at some time in the future, begin to reduce the health of the individual plants, depending largely on environmental factors (e.g., Sieber 2007). The same is true for arthropods and other animal pests. These native pests and pathogens are normal components in forest ecosystems. Being long-lived organisms, trees have developed strategies during evolution to cope with most potential pests and pathogens (Pearce 1996, Ayles et al. 2010).

Different problems arise when damaging invasive alien species (IAS) establish in the forests, however, as there have been no evolutionary pressures for the local trees to adapt to the presence of these organisms (Paap et al. 2020). A potential consequence, therefore, is that trees within the ecosystem are badly affected, possibly even dying because of attacks by these invasive species. Many examples of IAS are known in Europe (e.g., Santini et al. 2013), some of the most dramatic of which are detailed in the examples given below.

The history of the spread of plant pests and pathogens is closely intertwined with the development of shipping during human history (Liebhold et al. 2012, Santini et al. 2018, Costello et al. 2022). The desire to migrate developed early in human existence presumably during evolution in Southern Africa, undoubtedly driven by the need to access good food and water supplies, although it is possible that discovering what was over the next horizon was an exciting prospect too. Presumably the first migrations were on foot, but crossing rivers and, eventually, large expanses of water proceeded with the crafting of simple boats. Following the addition of sails, simple vessels could be used in passing over rivers and lakes and, possibly, were even used in migrations as far as from Europe to North America (Bill 2008).

Shipping evolved slowly, from small, canoe/kayak-like craft to the harnessing of wind power with sails, developing into the faster, ocean-crossing vessels of Mediaeval and later times. Towards the end of the 18th and into the 19th century, sails were supplemented with steam engines, followed by diesel engines in the early 20th century (Paine

Figure 1. Healthy forest ecosystems. **a)** *Picea abies*-dominated forest with some *Larix decidua* in the Dolomitic Alps, Northern Italy; **b)** A view over Yosemite National Park in the Sierra Nevada, California; **c)** *Fagus sylvatica* canopy in spring (Austria); **d)** Mixed coniferous forest to the south of Kaş, Turkey.

2014). The need to raise cereal and other crop plants in newly colonised areas led these early migratory humans to carry seed with them as they travelled. As seed are well-known vectors of many pathogens (Shade et al. 2017, Cleary et al. 2019), the diseases were transported along with the crops (Stukenbrock and McDonald 2008).

Although tree seed are well-known sources of certain pathogens (Cleary et al. 2019, Franić et al. 2019) major changes in the impacts of pests and pathogens came to the fore with the development of more efficient shipping. Reducing times at sea from months to a few weeks and less meant live plants, rather than seed, could survive long journeys.

In the past 70 years, shipping efficiency for goods has increased massively through the wide-spread adoption of containerisation. As containerised transport became more accepted, ships were built to carry larger numbers of containers on each trip. Currently, the largest container ship on the oceans is The Mediterranean Shipping Company (MSC) Gülsen, launched in 2019, with a capacity of nearly 24,000 standardised containers.

Although it is unlikely in practice, each container on a ship could include live plant materials. Within plant batches, each plant could potentially carry one or more cryptic pest or pathogen infestations. Many plants with compost may include more than one potential pathogen within the compost, possibly infecting the plant or simply living saprotrophically or as viable inoculum in the compost itself. Recent work suggested that individual plants can harbour multiple Oomycetes in the rhizosphere without any symptoms being visible (Puertolas et al. 2021). Imported plants are often grown on further in nurseries in the receiving state, before sale for landscaping or garden use. When the contaminated stock is planted out, the pathogens are introduced into a previously naïve environment, where they may establish on suitable host species in the newly invaded area, ultimately leading to major health issues on those plants and in those ecosystems (Jung et al. 2016).

Global trade in plants is a very complex system (see Santini et al. 2018), driven by the need for plants in landscapes and demand for different plants for gardens. Plant production systems in horticulture have also advanced greatly in the past 50 years (e.g., Davies et al. 2017). Currently, many woody plants are reproduced clonally by specialist growers outside Europe (for example; see FAO 2012). The young, rooted cuttings are then imported by European nurseries, where they are grown on, in pots of increasing size, before sale to the end users (Santini et al. 2018). Not all plants are produced in this manner: many native trees, for example, are raised in other specialist nurseries in Europe, sometimes for several years and either sold as root-balled specimens or raised into very large pots, before sale to end-users (e.g., Newman and Hayden-Smith 2014).

Examples of Serious IAS Causing Damage to Forest Ecosystems

There are many examples of tree pathogens causing wide-spread problems on a global scale. It can take several years following the likely introduction of the IAS for the problem to be noticed (e.g., Wylder et al. 2019). This period is the establishment phase for the IAS: it arrives as inoculum, and subsequently the population gradually builds up on the local plants it is able to colonise and reproduce from.

In the following section a number of these invasive pathogen problems are detailed, particularly those affecting forestry in Europe, but many others are described in the literature (e.g. Santini et al. 2013, Ghelardini et al. 2017). The choice of species was based on the extent of damage already caused and the potential to cause further damage with wider dissemination and climate change.

Phytophthora Species

Oomycota in genera including *Phytophthora, Pythium, Phytopythium*, *Saprolegnia Leptolegnia, Aphanomyces* and *Achlya*, are notorious pathogens of plants and animals, causing massive losses globally in agriculture, horticulture, aquaculture and natural ecosystems (Mendoza and Vilela 2013, Van West and Beakes 2014). Several species of *Phytophthora* are causing ongoing epidemics affecting trees in various regions of the world, with the most well-known species overall arguably being *Phytophthora cinnamomi* Rands and *Phytophthora ramorum* S. Werres, A.W.A.M. de Cook & W.A. Man in't Veld (Hansen 2015). There are at least 140 species of *Phytophthora* that are fully described in the literature (Scott et al. 2013, Jung et al. 2015), but it has been predicted that there could be at least 400-600 species in total (Brasier 2009). It is well established that *Phytophthora* and other plant pathogenic oomycetes are commonly transported in the 'plants for planting pathway'; recently published work demonstrated that individual hardy woody ornamental plants traded in garden centres and online almost all include one or more potentially pathogenic oomycetes on the plants or in the accompanying compost (Puertolas et al. 2021).

Phytophthora cinnamomi is rated as one of the worst plant pathogens known (Burgess et al. 2017), with a host list including over 1,000 plants. Serious problems can arise anywhere the pathogen is known, but hot spots of activity occur, some of which are associated with regions of high overall biodiversity. The pathogen probably evolved in Celebes, possibly including Taiwan (Ko et al. 1978), and was first discovered in the 1920s when cinnamon trees planted in Papua New Guinea began to die (Rands 1922, Shepherd 1975, Arentz and Simpson 1986). Some evidence suggests that *P. cinnamomi* had already been spread from its centre of origin before it was first isolated and described (e.g. Santini et al. 2013). Phytophthora dieback, caused by both *P. cinnamomi* and *P. x cambivora* (Petri) Buisman is a well-known problem on *Castanea sativa* Mill. in Europe, but *P. cinnamomi* also causes dieback and mortality on *Quercus* species (Figure 2a). The pathogen is considered to cause epidemics on *C. sativa* and *Quercus suber* L. throughout the Mediterranean region, with particular hot spots in Italy (Vannini and Vettraino 2001, Scanu et al. 2013), Spain (Rodríguez-Molina et al. 2005), Portugal (Brasier et al. 1993, Moreira and Martins 2005) and Turkey (Balcı and Halmschlager, 2003, Akıllı et al. 2012).

There is particular concern over the activity of *P. cinnamomi* and other *Phytophthora* species in UNESCO

biodiversity communities in South-Western Australia (Figure 2b) and in the Fynbos, South Africa, with extensive dieback of the woody flora, particularly on disturbed sites (Engelbrecht and Van den Berg 2013, Reeksting et al. 2014). In the past, *P. cinnamomi* caused an epidemic of little leaf disease on pines planted on nutrient depleted, former cotton growing sites in the southern USA (Copeland 1952, Roth 1954).

Warm temperatures of 25°C+, i.e., a Mediterranean climate, favour disease, particularly when associated with intermittent rainfall and droughts (e.g., Burgess et al. 2017).

a)

b)

Figure 2. Impacts of *Phytophthora cinnamomi* infection on: **a)** *Quercus suber* in Sardinia (photo: T. Jung); **b)** *Eucalyptus marginata* in Western Australia.

Canker of Sweet Chestnut

In the early 20th Century, serious dieback of the American chestnut, *Castanea dentata* (Marsh.) Borkh. was observed in the Bronx Zoological Garden, New York. The disease symptoms included formation of sunken cankers where the pathogen killed host tissues, with shoots distal to the infection dying; in many infections, the roots of the tree remain alive, but the top of the tree dies back completely, resulting in repeated regrowth from stump sprouts (Anagnostakis 1987). Investigations showed that the problem was caused by a fungal pathogen; the fungus was first named *Diaporthe parasitica* Murrill. (Murrill 1904, 1908) but later placed in the genus *Endothia* (Anderson and Anderson 1912). Subsequently the pathogen was reclassified as *Cryphonectria parasitica* (Barr 1978). Infections resulted in the formation of severe cankers on branches and the main stem (Figure 3), leading to dieback and mortality. In 1912-14, expeditions were launched to find the source of the pathogen and the same fungus was quickly discovered causing mild symptoms on *Castanea* species native to Japan and China, such as *C. mollissima* Blume and *C. seguinii* Dode (e.g., Fairchild 1923) from where infected chestnut timber was exported to North America (Anagostakis 1987).

The speed of spread of *Cryphonectria parasitica* in North America was alarming. The native chestnut there, *Castanea dentata*, occurred naturally throughout the Appalachian Mountains and in small pockets of woodland further south. It was a very important, multi-purpose tree species, with excellent timber qualities for many uses from fine furniture to railway sleepers, a durable bark which was used for roofing and, of course, the chestnuts themselves, which as with the Europe sweet chestnut, were highly valued for culinary purposes and also consumed by many wild animals in the native forests (Rigling and Prospero 2018). Within 40 years of *Cryphonectria parasitica* being recorded in the Bronx Zoological Gardens, New York, the pathogen had spread throughout the native range of *Castanea dentata*, killing most trees or reducing them to small shrubs (Anagostakis 1987).

In the last ten years, chestnut trees, derived by hybridising *C. dentata* with *Castanea* species from Far East Asia which showed good levels of resistance to the pathogen, are now being planted out in the Eastern USA (Clark et al. 2019). A major objective of the breeding programme was to produce trees that had a growth habit as close to *C. dentata* as possible. A recovery programme is now underway in the Appalachians, but it took over 100 years to reach that point from the time the disease was first noticed.

The situation in Europe differed from that in North America, however. The presence of *Cryphonectria parasitica* was first recorded in Italy in 1938 (Biraghi 1946), with observations of dieback of the European sweet chestnut, *Castanea sativa*. Regular surveys for the disease were made after World War II and, in the early 1950s Biraghi (1953) reported that cankers recorded in previous surveys appeared to be reducing in size, enabling trees to partially recover from the infections. It took over 10 years to prove that this effect was due to the presence of a virus-like infection in *Cryphonectria parasitica*, causing hypovirulence (low virulence) in infected strains of the pathogen (Grente

Figure 3. Symptoms of *Cryphonectria parasitica* attacking *Castanea sativa*: **a)** well-formed canker on stem of *C. sativa* in northeast Turkey; **b)** canker on branch; **c)** canker caused by virus-infected hypovirulent strain of *C. parasitica*; **d)** early symptoms of canker, causing death of branches (photos c and d: Kiril Sotirovski).

1965). This hypovirulence has been exploited in many European countries and in Turkey (see Rigling and Prospero 2018) to reduce the impacts of chestnut canker in nut and timber-producing regions. Hypovirulence works well in Europe and Eurasia, where genetic diversity in the pathogen is low, compared to the higher relative diversity in North America (MacDonald and Fulbright 1991, Liu et al. 1996). Many European countries have used extended programmes to investigate the efficacy, longevity and non-target impacts of hypovirulent strains of the pathogen (Robin et al. 2000, Krstin et al. 2017, Diamandis 2018). There have also been trials of new formulations and delivery systems for the hypovirulence factor (Kunova et al. 2016). Currently, the disease continues to spread in Europe, including into the UK (Hunter et al. 2013), with attempts to use hypovirulence as the major management tool widespread in affected parts of the continent.

Dutch Elm Disease

A striking example of the potentially catastrophic impacts of invasive alien pathogens on tree populations in recent history was the spread of the Dutch elm disease pathogen, *Ophiostoma novo-ulmi* Brasier, firstly in the 1930s in North America, then from the early 1960s in Western Europe (Brasier and Gibbs 1973). There was a previous epidemic of Dutch elm disease in northern Europe in the early 20th Century, which caused considerable dieback in the *Ulmus* populations and led to mortality in approximately 30% of the elms present at the time (Peace 1962). The pathogen was defined as *Ceratocystis ulmi* (Buism.) (later: *Ophiostoma ulmi* (Buism.) Melin & Nannf. [1934]) (Gemma 1984). By the 1950s, however, that epidemic had run its course (Peace 1962); but within a few years, it was noted that elms in parts of England were dying. Mapping of the outbreak showed that deaths were focused around major ports in England

and Wales, and an investigation of *Ulmus thomasii* Sarg. logs imported from Canada into southern England demonstrated the presence of the Dutch elm disease pathogen, along with North America elm bark beetles, *Hylurgopinus rufipes* (Eichhoff) (Strobel and Lanier 1981) (Coleoptera: Scolytidae) in the timber consignments (Brasier and Gibbs 1973). Bark beetles are vectors of the Dutch elm disease pathogens; in Europe, the vectors include several species of *Scolytus*, mainly *Scolytus scolytus* Fabr. (Coleoptera: Curculionidae) but replaced by *Scolytus multistriatus* (Marsham 1802) (Coleoptera: Curculionidae) in more northerly latitudes (Santini and Faccioli 2015). In depth analyses showed that the second outbreak of Dutch elm disease was caused by a previously unknown species, subsequently named *O. novo-ulmi* (Brasier 1991, 2000). There is an interesting phenomenon occurring: into the late 1970s, it was relatively easy to isolate *O. ulmi*, the pathogen which caused the early 20th century outbreak of Dutch elm disease from stands of elm where dieback was occurring. More recently, however, it has become rare to find *O. ulmi* in isolations from similar niches. It appears that the causal agent of the later Dutch elm disease outbreak has replaced the original pathogen, possibly driving it to extinction (Brasier 2000).

European and North America species of *Ulmus* are all highly susceptible to infection by *O. novo-ulmi* (Brasier 2000). The disease cycle is heavily reliant on the elm bark beetle vector, on which the spores of the pathogen adhere to the beetle exoskeleton (Webber 2000, Santini and Faccioli 2015). Elm bark beetles target trees in decline for egg laying (Webber and Brasier 1984). Following mating, the females bore a brood gallery into bark on declining elms, laying eggs at regular intervals along the gallery. When the eggs hatch, the larvae consume the nutritious secondary phloem of the inner bark tissues, with the pattern of galleries formed varying between *Scolytus* species (Figure 4a). Following pupation, the newly emerged beetles exit the dead bark tissues, but before mating must carry out maturation feeding, usually on bark in the upper parts of a healthy elm crown, often in branch crotches (Webber and Brasier 1984; Figure 4b). When emerging from the old bark tissues, the young beetles are in an environment that is perfect, in terms of temperature and humidity, for the pathogen to produce large numbers of coremia (fused conidiophores) covered in sticky masses of asexual spores. These spores adhere to the beetle exoskeleton and, when the beetles begin maturation feeding, the spores are introduced into the wounds formed, gaining entry to the host vascular tissues (Figure 4d). The tree is then infected: brown staining in the xylem tissues, typical of vascular wilt diseases, forms as the tree responds to the damage. Toxins produced by the fungus, however, cause severe disruption to the tree water balance, causing stomata to stay open and tyloses to form in the xylem vessels, even those that are not directly infected by *O. novo-ulmi*. The tree wilts and, eventually, dies. Bark beetles are attracted to the dying trees, lay their eggs in the bark tissues, and the disease cycle begins again (Webber and Brasier 1984).

Xylella fastidiosa

The problems caused by *Xylella fastidiosa* Wells et al. 1987, a xylem-limited bacterium, were recognised in North America in the late 19th Century, when a severe problem arose on grapevines planted for viticulture in California (Jeger and Bragard 2018). The US Department of Agriculture sent a 'special agent' to investigate the problem: Newton B. Pierce became the first professional plant pathologist working in California, and demonstrated that the unknown cause of the disease on grapevines was transmitted by sap sucking insects. The agent responsible, however, remained unknown until the early 1970s, when an unculturable species of xylem-limited bacterium was suggested as the cause (Goheen et al. 1973, Hopkins and Mollenhauer 1973). The causal agent was partially described from grapevines (Davis et al. 1978) and fully described as *X. fastidiosa* by Wells et al. (1987).

Subsequently, it was shown that at least four, possibly six, distinct sub-species of *X. fastidiosa* occur, with differing host ranges (Nunney et al. 2010, 2012), including *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *pauca* and *X. fastidiosa* subsp. *sandyi*: all subspecies are native to the American continents.

Collectively, *X. fastidiosa* sub-species cause dieback and mortality on a very wide range of woody plants, with, so far, almost 600 hosts known to be infected (European Food Safety Authority 2020). Most *X. fastidiosa* disease reports are from North and South America, usually in tropical and sub-tropical climates (Desprez-Loustau et al. 2021), although there are reports from Canada (Goodwin and Zhang 1997). According to the EFSA database, 15 forest tree species common in European forests are known hosts of *X. fastidiosa*, of which nine have been recorded as infected in Europe. Some affected plants that are widely planted in Europe, such as *A. platanoides* L. and *Q. rubra*, have not yet been confirmed to be infected. *Xylella fastidiosa* is currently restricted to Mediterranean Europe, where these species are uncommon (Desprez-Loustau et al. 2021). A current notable problem of relevance to south-eastern Europe and beyond is that of 'olive quick decline syndrome' occurring in olive groves in Apulia, Southern Italy (Elbeaino et al. 2014, Loconsole et al. 2014). Spread from the infected area is considered highly likely, threatening a disastrous impact on olive production throughout the Mediterranean basin. Further identifications of *X. fastidiosa* in Europe in recent years suggest that several invasive events have occurred (Baldi and La Porta 2017). It is feared that further spread of the *X. fastidiosa* strains now known to be present in Europe could lead to serious problems on many other species of woody plants, in horticultural production, in gardens and in forests and woodlands (Jeger and Bragard 2019, Morelli et al. 2021).

Ash Dieback

The pathogen causing dieback of ash (*Fraxinus* spp.) in Europe, *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya,, is native in east Asia, including Japan and the far eastern regions of China and Russia, where it is considered a saprotroph on species of *Fraxinus* native in that region (Gross et al. 2014, Zhao et al. 2012). The first indication of serious problems on ash in Europe came in the early 1990s, when a progressive dieback was noted on *Fraxinus excelsior* L. in the north east of Poland and the south west of Lithuania (Przybył 2002, Gil et al. 2006, Kowalski 2006). The problem
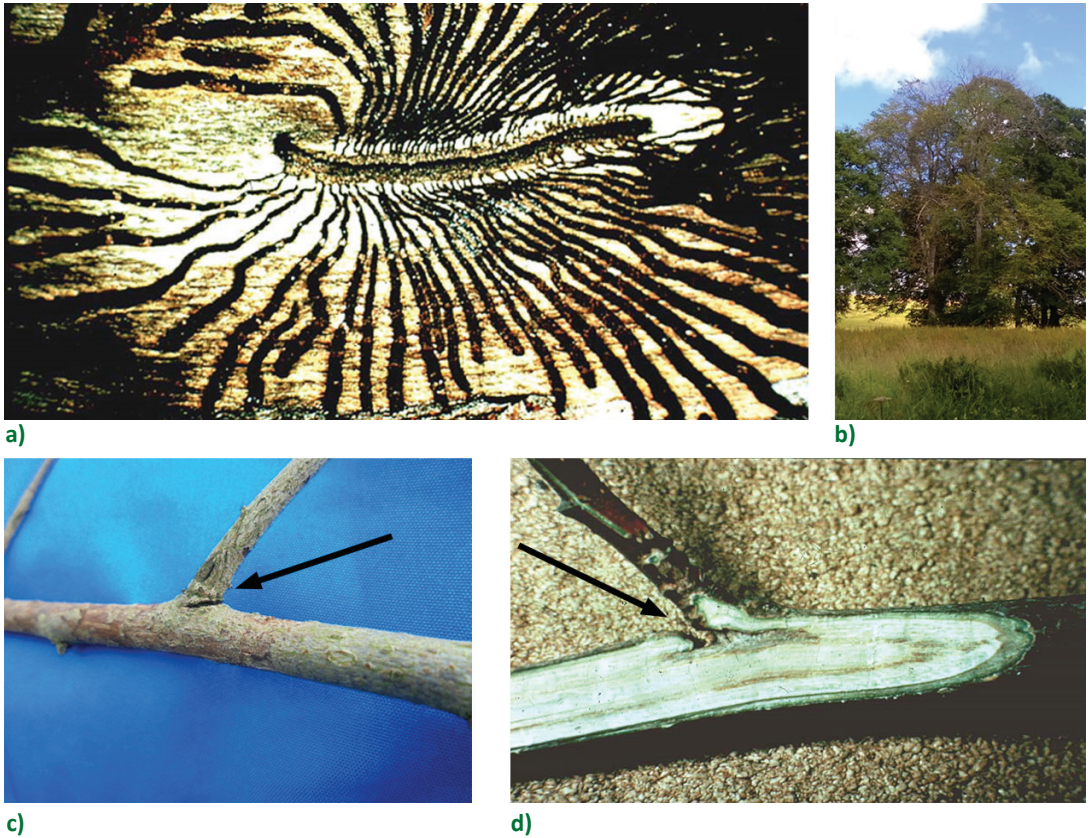
Figure 4. Typical symptoms of Dutch elm disease: **a)** underside of bark removed from the lower stem of *Ulmus minor* with breeding gallery of one of the vector beetles, *Scolytus scolytus*; **b)** large *U glabra* trees in a field boundary dying of *O. novo-ulmi* infection; **c)** notch (arrowed) created during maturation feeding by the vector beetle; **d)** typical staining in the xylem of a twig, following maturation feeding by *S. scolytus* (photos a and d, courtesy of Don Barrett).

had spread more widely in Europe before the causal agent was identified definitively in the mid-2000s (Baral et al. 2014), when it had already caused great amounts of damage in eastern and central Europe, and into western Europe. The pathogen appears to infect all *F. excelsior* (Figure 5) and *Fraxinus angustifolia* Vahl. trees, although individual host genotypes vary greatly in the symptoms shown (Krautler and Kirisits 2012). A third *Fraxinus* species native in Europe, common in the Balkans, *Fraxinus ornus* L. may be infected but shows limited symptoms, compared with *F. excelsior* and *F. angustifolia* (Kirisits and Schwanda 2015). *Fraxinus* spp. from Far East Asia are considered resistant to the disease, although foliage is infected: it appears that a difference between the two highly susceptible European ash and those in the Far East of Asia is that the fungus fails to grow from the leaf petioles and in to the twigs and branches of the Asian species (Cleary et al. 2016). The mechanisms underlying this difference are under investigation.

Box Blight

Box (boxwood) blight was first reported from infected samples in England in 1994 (Henricot and Culham 2002,

Henricot 2006), causing dieback of ornamental *Buxus sempervirens* L. clones, since when the problem has been reported from all over the world where the plant is used extensively in amenity and garden plantings (see Leblanc et al. 2018). Apart from ornamental settings, the disease is killing *B. sempervirens* (and the putative species, *B. colchica*) in the wild in some European and Eurasian countries. In Turkey, for example, where *B. sempervirens* is the dominant understory species in forests of the north-eastern Black Sea region, as many as 90% of certain box populations lost all foliage within 12 months of the first report of the disease occurrence (Figure 6; Akilli et al. 2012, Mirabolfathy et al. 2013, Lehtijärvi et al. 2014, 2017). Conditions in the Black Sea region of Turkey and neighbouring Georgia, with high humidity most of the time, are perfect for disease development and spread of the spores.

The first report of the disease in the USA was in 2011, but by 2018 it was known in 25 states (LeBlanc et al. 2018).

The disease is caused by two similar species, *Calonectria pseudonaviculata* L. and *Calonectria henricotiae* sp. nov., first classified as *Cylindrocladium*. The origins of these fungi are unknown, but it is believed that they are spread by human

a)

b)

Figure 5. Symptoms of ash dieback caused by *Hymenoscyphus fraxineus*: **a)** Young ash tree with severe dieback on many branches; **b)** Typical premature death of severely infected foliage, which remains attached to the tree for some time.

activities, particularly through the transport of live plants (LeBlanc et al. 2018). Coupled with the activities of the box moth, *Cydalima perpectalis* (Walker, 1859), inadvertently imported into Europe and Eurasia from Asia (Leuthardt et al. 2010, Nacambo et al. 2014, Bras et al. 2019), the future of *B. sempervirens* in both native forest areas and in ornamental plantings is in serious doubt.

Canker Stain of *Platanus*

The pathogen, *Ceratocystis platani* (Walter) Engelbrecht & Harrington, causing this lethal disease of oriental and hybrid plane in Europe is probably native to the southeast of North America (Engelbrecht et al. 2004) but was accidentally introduced into Europe during World War II (Panconesi 1999). The western plane, *Platanus occidentalis* L., is susceptible to infection by *C. platani*, but the disease caused is a more mild, often trivial, dieback than occurs on *Platanus* x *hispanica* Mill. or *Platanus orientalis* L. The common clones of *P.* x *hispanica* are, almost without exception, very susceptible to infection and will die within months, regardless of tree size (Ferrari and Pichenot 1976, Panconesi 1999). The plane species native in parts of south-east Europe and Turkey, *P. orientalis*, is highly susceptible and infection invariably leads to death. With *P. orientalis* being a major component of riparian ecosystems in the eastern Mediterranean region, as well as a very important urban shade tree, the threat posed by the canker stain pathogen is enormous (Tsopelas et al. 2017).

To date, problems caused by *C. platani* are reported in Italy (Panconesi 1972), Switzerland (Panconesi 1999), France
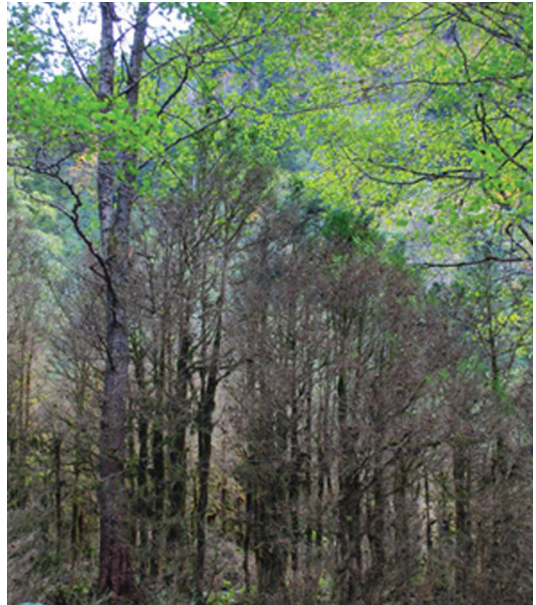

Figure 6. *Buxus sempervirens* trees forming the main understory of natural forests in The Black Sea region of Turkey. The trees are dying due to severe blight attack: note the very tops of the trees remain green, in a niche that is better ventilated than nearer the ground. (Photo: Asko Lehtijarvi).

(Ferrari and Pichenot 1974, 1976, Vigouroux 1986), Greece (Tsopelas and Angelopoulos 2004), Albania (Tsopelas et al. 2015) and Turkey (Lehtijarvi et al. 2018). After entering Italy, the problem was first noted when urban *Platanus* trees, mostly *P. x hispanica*, began dying, spoiling the aesthetic qualities of major avenues (Figure 7) comprised entirely of plane (Panconesi 1999). Subsequently, the pathogen spread into south-east France before migrating northwards, extending as far as Switzerland. With a few possible exceptions, all of the trees affected in Italy, France and Switzerland were planted: the only native stands of plane (*P. orientalis*) in Italy were restricted to a few small sites in Sicily (Tutin 1964).

In the early 2000s, a serious dieback and death of *P. orientalis* began in native stands in Greece and was discovered to be caused *C. platani* (Tsopelas and Angelopoulos 2004, Tsopelas and Soulioti 2011). It is thought that the pathogen was inadvertently taken into Greece on plane trees imported from nurseries in Italy. Subsequently, the disease was also found in the south of Albania, near to the border with Greece (Tsopelas et al. 2015). Currently, the disease appears to have

spread throughout much of Albania and is killing *P. orientalis* in river valleys towards the eastern border with North Macedonia (Woodward and Doğmuş, personal observations, September 2021). Canker stain is also killing *Platanus* trees in Istanbul, where the hybrid and *P. orientalis* are iconic features of the landscape and constitute major proportions of the urban tree population (Lehtijarvi et al. 2018). An additional complication is that a second lineage of *C. platani* now appears to be in the Mediterranean region, possibly leading to changes in virulence and further loss of *Platanus* trees (N. Soulioti, personal communication, April 2022).

Canker stain of plane resembles a wilt disease: the pathogen enters the tree through wounds, often caused by pruning in urban areas, and grows rapidly in the xylem of hosts. Toxins released by the growing fungus have a catastrophic impact on water balance in the infected trees, causing rapid water loss through open stomata, wilting, dieback and death (Panconesi 1981). Due to routine pruning activities in urban areas, the pathogen can spread rapidly from tree-to-tree, leading to serious impacts on the aesthetics of street and park plantings.

Figure 7. Symptoms of *Ceratocystis platani* infections on *Platanus* trees: **a)** infected *P. x hispanica* dying in a street in Firenze, with an apparently healthy tree to the left and a symptomatic tree to the right; **b)** avenue of *Platanus* dying on a major road in Istanbul; **c)** young, riparian *P. orientalis* dying from infections; **d)** cross-section through a stem showing typical internal staining of the wood following infection. (photo b: Asko Lehtijarvi; d: Panghiotis Tsopelas/Nikoleta Soulioti).

Dothistroma Needle Blight (DNB)

Dothistroma species have been recognised pathogens impacting on growth of most species of *Pinus* for many years, with the first record dating back to 1910, in the north-west of Russia (Doroguine 1911, Drenkhan et al. 2016). Plantations of *P. radiata* D. Don. in East Africa were devastated by the disease in the 1950s and 1960s, making planting pines in that region almost hopeless (Gibson 1974). *Pinus radiata* plantations in New Zealand were also very badly damaged around the same time, but techniques for reducing the damaging effects were heavily researched and put in place to maintain the stock of *P. radiata* which, diseases notwithstanding, grows exceptionally well in the region (Rogers 2002). Until the 1990s, therefore, DNB was considered a problem in plantation pines in the southern hemisphere. In the early 1990s, however, natural pinewoods and plantations in Europe and North America were attacked by DNB, causing massive losses in several *Pinus* spp. (see Drenkhan et al. 2016). Research in South Africa showed that what was considered a single pathogen, *Dothistroma pini*, was, in fact, two cryptic species, *D. pini* Hulbary and *D. septosporum* (Dorog.) Morelet (Barnes et al. 2004), of which *D. septosporum* seemed to be the more common globally and the species responsible for the large DNB outbreaks in pine forests of much of the Northern Hemisphere. Species of pine particularly badly damaged in their natural habitats by *D. septosporum* and *D. pini* include most sub-species of *P. nigra*, certain provenances of *P. contorta* Douglas and *P. brutia* Ten. and *P. jeffreyi* Balf. Climate plays a large part in determining the disease severity, however, resulting in sporadic epidemics (Woods et al. 2005). Dothistroma species are known to infect 109 hosts, 95 of which are in the genus *Pinus*. *Dothistroma septosporum* has also been recorded infecting species of *Abies, Cedrus, Larix, Picea* and *Pseudostuga*, all genera within the Pinaceae (Drenkhan et al. 2016).

The massive upsurge in incidence and severity of Dothistroma needle blight recognized from the early 1990s onwards, differs from the changes in invasive alien pathogens described for the other selected pathogens because it is possible that the causal agents, *D. septosporum* and *D. pini*, were widespread in North America and Northern Europe prior to the epidemic outbreaks we are now witnessing (Drenkhan et al. 2016). Although the centres of evolution of the pathogens are currently unknown, recent work demonstrated that there is great genetic diversity in *D. septosporum* populations in Turkey (Oskay et al. 2020b), suggesting that this region could be amongst the centres of evolution for this pathogen. As a region bridging Europe and Asia, although in the absence of large numbers of isolates of the pathogen from Asia for use in comparisons, it is difficult to assess fully this hypothesis. Spread of different genotypes of the pathogen also plays a role in the increased Dothistroma damage seen in Europe (Ennos et al. 2020).

As the common name suggests, DNB causes lesions on the needles (Figure 8), sometimes leading to defoliation and death. The common name prior to the upsurge in the epidemic was 'Red Band Needle Blight', referring to a symptom on infected needles, mainly of *P. radiata* (Gibson 1972); the red banding does not appear on all species of pine, or even on all infected individuals within a species

hence the change in common name from the late 1990s onwards. A notable effect of the disease on surviving trees was the significant reduction in the number of years foliage retained on affected pines (Drenkhan et al. 2016), resulting in the trees having much thinner crowns than prior to the 1990s.

a)   b)

Figure 8. Symptoms of Dothistroma needle blight on: **a)** *Pinus brutia* in western Turkey; **b)** needles of *Pinus jeffreyi*. (photo a: Asko Lehtijarvi).

Brown Spot Needle Blight of Pines

Lecanosticta acicola (Thüm.) Syd. (syn. *Scirrhia acicola*; *Mycosphaerella dearnesii*) causes brown spot needle disease of *Pinus* spp., and can be particularly problematic in Christmas tree plantations. Disease symptoms include brown lesions on infected needles (Figure 9a), which can lead to early loss of foliage and, in severe or repeated cases, death of the affected tree (Figure 9b). The pathogen is widespread in North America, where it is believed to be native, but has spread into Central and South America, the Far East of Asia, Europe and Turkey (https://gd.eppo.int/taxon/SCIRAC/distribution). Infections have been confirmed on over 40 *Pinus* species (see: van der Nest et al. 2019), plus several sub-species and hybrids, along with *Cedrus libani* (Oskay et al. 2020a).

It appears that *L. acicola* has spread rapidly in Europe over the last 15 – 20 years, with many reports from different countries in the region (Van der Nest et al. 2019), although to date, it has not been reported from the southern Balkans. With multiple threats to pine vigour and productivity, *L. acicola* presents a further worrying disease affecting this important genus of trees.

Cypress Canker and Dieback

The first epidemic of cypress canker and dieback disease caused by *Seiridium cardinale* (W.W. Wagener) B. Sutton & I.A.S. Gibson. *Seiridium cardinale* was reported on *Cupressus macrocarpa* Hartw. on the Monterey Peninsula, California (Wagener 1928), but the disease subsequently spread in the early to mid-twentieth century

a)
b)

Figure 9. Symptoms of brown spot needle blight caused by *Lecanosticta acicula*: **a)** needles of *Pinus nigra* subsp. *nigra* showing characteristic spots; **b)** *P. nigra* subsp. *pallasiana var. pallasiana* f. şeneriana, taken in the Atatürk Arboretum, Istanbul in 2018; within two years, this ornamental tree was dead. (photos: Funda Oskay).

to Australia and New Zealand, Europe and South America, via movement of infected plant material (Birch 1933, Barthelet and Vinot 1944, Grasso 1951, Saravi-Cisneos 1953, Anastassiadis 1963, Mujica et al. 1980, Torres 1969, Funk 1974, Caetano et al. 1991, Wingfield and Swart 1988, Danti et al. 2009). The disease has been reported on species of *Cupressus, Chamaecyparis, Cryptomeria, Juniperus, Thuja* and *×Cupressocyparis* (Anastassiadis 1963, Graniti 1998).

This disease problem was first recognised in Europe when an outbreak occurred in Italy in the 1930s (Graniti 1998). Once in southern Europe, however, *Cupressus sempervirens* L., an iconic tree species in Mediterranean landscapes proved highly susceptible to infection and damage from *S. cardinale* (Figure 10). In many regions now, the relatively non-susceptible species, *Cupressus arizonica* Greene is used as a replacement tree with similar environmental tolerance, although there is no obvious substitute for the fastigiate form of *C. sempervirens* (Beresford and Mulholland 1982, Saos et al 2001).

A number of other invasive pathogens are present in Europe, although spread to date has been limited. Examples include pine pitch canker, caused by *Fusarium circinatum* Nirenberg & O'Donnell, which is established in Portugal and Spain: outbreaks in France and Italy are considered to be eradicated (Wingfield et al. 2008, Drenkhan et al. 2020). The pine wilt nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is native to North America, but established in China and

Japan early in the 20th Century and was diagnosed causing death of *Pinus pinaster* in Portugal in the late 20th Century (Mota et al. 1999). Any further spread and establishment of these highly damaging problematic organisms from the Iberian Peninsula to other parts of Europe will result in extensive damage to pines and blocks trade in pine timber from invaded territories.

Influence of Climate Change

Although global trade is undoubtedly the main factor in the increasing invasions of forests and ornamental plantings by damaging alien pests and pathogens (Santini et al. 2018, Pyšek et al. 2020), climate change is also having a major influence on establishment and spread of these organisms, affecting pest and pathogen activities and life cycles, host plant physiology and the outcomes of interactions between pests and pathogens and the plants (Tubby and Webber 2010). Climate change, as suggested above for Dothistroma needle blight, is also altering the latitudes at which endemic pathogens are causing problems in forests (La Porta et al. 2008). Much of the climate effect is due to changing temperatures and humidities, altering the extent and timing of previous seasonal effects. Increasing frequencies of storm events and droughts also will have large effects on many pathogen life cycles (La Porta et al. 2008, Sturrock et al. 2011, Garrett et al. 2021).

Modelling has been carried out and published for several of the examples of invasive alien pathogens given

Figure 10. Symptoms of *Seiridium cardinale* infection on *Cupressus sempervirens*: **a)** top-dieback of a mature tree outside the centre of Firenze, Italy; **b)** flagging of smaller branches on a tree in an avenue in Italy.

above (e.g., Guerina et al. 2001, Harwood et al. 2011, Watt et al. 2011, Burgess et al. 2017, Danti and Della Rocca 2017, Möykkynen et al. 2017, White et al. 2017, Chumanová et al. 2019, Daughtrey 2019, Mesanza et al. 2021): below, we present summaries of the published models for two of the pathogens, *P. cinnamomi* and for *D. septosporum*, for which considerable information is available.

Phytophthora cinnamomi: This pathogen causes root and collar rot in many woody plant species resulting in a disease known as *Phytophthora* dieback (Cahill et al. 2008). As one of the most significant and damaging plant pathogens known (Kamoun et al. 2015, Hardham and Blackman 2018), the potential impact of predicted climate change on the distribution and activity of *P. cinnamomi* has been the subject of considerable attention (Brasier and Scott 1994, Bergot et al. 2004, Desprez-Loustau et al. 2007, Thompson et al. 2014, Duque-Lazo et al. 2016, Burgess et al. 2017). In the latest of these publications, Burgess et al. (2017) based the models on the known extensive global distribution of *P. cinnamomi*, utilizing the CLIMEX approach to climate change modelling. CLIMEX enables users to produce a likely scenario for pathogen (or pest) activity, given information on current knowledge of the distribution and environmental requirements of the organism; different climate change models can then be incorporated, enabling predictions of pest/pathogen distribution in the future.

Good conditions for the establishment of *P. cinnamomi*

include the presence of suitable host plants and conducive soils and a climate that includes alternating warm, wet winters with dry summers (Hardham and Blackman 2018), as found in regions with Mediterranean climate types globally. Predictions for climate change suggest that some of the regions currently affected by *P. cinnamomi* may, with time, become less suitable for the pathogen and a decrease in damage will be observed (Burgess et al. 2017). It is also likely, however, that climate change will lead to an increase in the latitudes and altitudes at which *P. cinnamomi* can establish and survive, with impacts in regions where this pathogen has hitherto not been a problem, including Canada and Scandinavia. Although the changes in climate suitability for *P. cinnamomi* to become active may result in greater damage in northerly latitudes, it is possible that regions around the Mediterranean Sea will become less suitable for the pathogen to be active. It is known that *P. cinnamomi* survives in northerly latitudes (e.g., Chavarriaga et al. 2007), possibly in the roots of symptomless host plants (Jung et al. 2013), indicating that the organism has already spread widely in the world, being transported into forests via infections in plant nurseries (Jung et al. 2015, Benavent-Celma et al. 2022).

Dothistroma septosporum: The global distributions of *D. septosporum* and *D. pini* are well-known (Drenkhan et al. 2016), along with the environmental requirements for DNB to occur and cause epidemics. Based on the available data, a number of models have been published to predict

the future spread and severity of *D. septosporum* (e.g., Watt et al. 2011, Möykkynen et al. 2017). These models are useful in determining the likely pattern of spread and regions where severity may increase in the future, along with predicting the effects of human interventions on the likely spread of the pathogens.

Using a model derived from a cell-based mechanistic approach, Möykkynen et al. (2017) simulated the spread of *D. septosporum* in Europe, assuming infected seedlings were the main sources of infective material, with further spread to trees of any age in the forest after planting; hence, the affected nurseries were assumed to be points of entry for the pathogen. Changing climate was included in the model utilizing ecoclimatic index maps for *D. septosporum*, derived from CLIMEX, using climate data from 1960 to 1990 as the baseline and future climate predicted using the IPCC (2007) scenario A1B.

This approach was supported by the proposal that the upsurge in DNB in the UK occurred following the transport of young pines between regions in the UK, resulting in the long-distance movement of more virulent strains of *D. septosporum* through the country (Mullett et al. 2017, Ennos et al. 2020).

Simulations derived from the model suggested that DNB will increase in incidence and severity in more northerly regions of Europe, whilst a decrease will occur further south, including in the Balkans peninsula. Some of the regions where disease is likely to increase include major pine-growing countries, such as Sweden and Finland. These predicted changes were related to the likely increases in rainfall and temperature during spring and summer months, generating the humidity required for spore dispersal and infection, as suggested elsewhere (Gadgil 1974, 1977, Woods et al. 2005, Dvorak et al. 2012, Welsh et al. 2014, Woods et al. 2016).

CONCLUSIONS

It is clear that, without the immediate imposition of more strict standards for the import and export of plants and plant materials on a global scale, invasions of potentially damaging alien pests and pathogens will continue. Under current global trade rules, it is difficult to impose tighter controls, due to the over-riding requirement for free trade whenever possible. Some steps in the direction of better controls have been imposed, however, such as the requirements for heat treatment of wood and timber (International Standards for Phytosanitary Measures No. 15 [ISPM 15]) to control possible transport of *B. xylophilus* and other wood-inhabiting organisms, and the far more stringent rules outlined in ISPM 36, which, partly at least, transfers responsibilities for exporting 'clean' stock to the producing nurseries and regions. Many individual states

are tightening up their plant import regulations too, in order to improve biosecurity.

Overall, climate change appears to be making the issues caused by invasive pests and pathogens worse than previously anticipated, although the activity of pathogens may reduce in certain regions, due to climatic changes that alter the suitability of different ecological zones for establishment and infections to occur (Sturrock et al. 2011, Burgess et al. 2017, Tank et al. 2021).

Considerable hope, however, lies in the enormous genetic 'power' in plants: humans could, given the political will, establish selection and breeding programmes that will ultimately enable us to maintain plantings of trees with far greater tolerance of changing climate and both endemic and invasive pests and pathogens (e.g., Sniezko 2006, Budde et al. 2016). Harnessing the 'omics' revolution will enable the application of marker assisted breeding, based on a much more thorough understanding of the physiological and molecular processes in plants that result in resistance to both biotic and abiotic problems. For example, a breeding approach was applied over many years in order to develop genotypes of *Castanea* with considerable resistance to *Cryphonectria parasitica* and in the past 15 years, clones of these resistant trees have been planted in the east of North America (Clark et al. 2019): it took over 100 years to get from the first record of *C. parasitica* and sweet chestnut canker in the USA to a point where resistant trees with similar growth forms to the American sweet chestnut could be planted out. There are also clones of *Ulmus* hybrids available that show resistance to the Dutch elm disease pathogen, *O. novo-ulmi*, with little sign of the resistance breaking down, as can happen when single or oligomeric resistance is deployed (e.g. Smalley and Guries 1993, Solla et al. 2005). Of course, with state-of-the-art technologies, the time line for introducing truly resistant clones of any tree species may be reduced considerably, as indicated in recent research on ash dieback, for example (Cross et al. 2017, Sambles et al. 2017, Stocks et al. 2019, Nemesio-Gorriz et al. 2020).

Author Contributions

SW, HTD and RCB contributed equally to the research, and writing.

Conflicts of Interest

The authors declare no conflict of interest.

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ORIGINAL SCIENTIFIC PAPER

Diversity of Ectomycorrhizal Fungi in Young Pedunculate Oak Stand from Morović, Serbia

Marina Milović[1]*, **Branislav Kovačević[1]**, **Saša Pekeč[1]**, **Andrej Pilipović[1]**, **Lazar Kesić[1]**, **Saša Orlović[1,]** **Anđelina Gavranović Markić[2]**

(1) Institute of Lowland Forestry and Environment, Antona Čehova 13d, RS-21000 Novi Sad, Serbia; (2) Croatian Forest Research Institute, Division of Genetics, Forest Tree Breeding and Seed Science, Cvjetno naselje 41, HR-10450 Jastrebarsko, Croatia

* **Correspondence:** e-mail: katanicm@uns.ac.rs

ABSTRACT

Although oaks belong to the economically most important hardwood tree species in Europe, data on the diversity of ectomycorrhizal (ECM) fungi on pedunculate oak in the Republic of Serbia are deficient. The aim of our study was to give the first insight into the diversity of ECM fungi in the young stand of pedunculate oak in Morović, Serbia. The combination of morpho-anatomical characterization and molecular analysis was used for determination of ECM fungi on pedunculate oak. Studied parameters included: (i) counting of vital ECM root tips, (ii) calculation of diversity indices and (iii) classification of morphotypes of ectomycorrhizae into the exploration types. Eighteen ECM fungal taxa were recorded in the studied young pedunculate oak stand. Seven of them were identified to the level of species, eight fungi to the genus, two to the family level, and one ECM fungus remained unidentified. ECM communities consisted of a small number of abundant taxa and a bigger number of rare taxa. The most abundant ECM fungi were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea*, which made association with the majority of ECM root tips. Short-distance exploration type (ET) dominated, followed by medium-distance smooth ET and contact ET, while long-distance ET and medium-distance fringe ET were rare at the Morović site. Values of diversity indices obtained in the studied pedunculate oak stand were comparable to the ones recorded in different oak stands over Europe. In order to get a deeper insight into the diversity of ECM fungi on pedunculate oak there is a need to continue research on increased number of sites and also to include seasonal dynamics.

Keywords: ectomycorrhizae; *Quercus robur* L.; morpho-anatomical characterization; ITS region

INTRODUCTION

Pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) are among the most economically important hardwood forest tree species in Europe (Ducousso and Bordacs 2004, Eaton et al. 2016, Kesić et al. 2018). They are light-demanding trees that enrich forest biodiversity by affecting the regeneration of many other tree species (Eaton et al. 2016). According to Eaton et al. (2016), fertile and well-watered soils are preferred by pedunculate oak, although adult trees tolerate floodings (Eaton et al. 2016).

In the Republic of Serbia penduculate oak covers 32400 ha of the land, which makes up to 1.4% of the total forest area (Banković et al. 2009) with the largest areas in the valleys of the Sava, Danube, and Morava rivers, while the best-quality pedunculate oak forests are situated in the Srem District (Rađević et al. 2020). However, oak trees in lowland forests are under detrimental influence of climate change and pedunculate oak is a species especially sensitive to precipitation and temperature (Kostić et al. 2019).

In most temperate ecosystems, the establishment, growth and survival of forest trees depend on mutualistic association with ectomycorrhizal (ECM) fungi. ECM fungi are successful in the absorption of water and nutrients from the soil and their translocation to plants, while plants provide fungi with carbohydrates (Smith and Read 2008). Common

mycelial networks connect plants of the same and different species with decomposers in the forest soil and can affect the ecophysiology of plants (Selosse et al. 2006). The importance of ECM refers to their supportive role in forest regeneration, succession and resilience to stress factors (Selosse et al. 2006). Particularly, trees that are under environmental stress benefit from the stabilizing effects of mycorrhizas (Milović et al. 2021a, Stojanović et al. 2021).

The functional compatibility of the partners in ectomycorrhiza is species-specific and depends on both partners (Kraigher 1996). Pedunculate oak is the host of Italian white truffle (*Tuber magnatum* Pico) which is the most expensive fungal species in the world, and it cannot produce fruit bodies without its host (Katanić et al. 2017). Not only that knowledge about the structure of the ECM community could give important information about the physiology of forest trees, but it can also reveal a lot about the functioning of forest ecosystems (Kraigher et al. 2011).

Considering the scarcity of the data on the ECM diversity of the oaks in the Republic of Serbia, especially those from underground studies of ECM root tips, this study aimed to give the first insight into the diversity of ECM fungi on pedunculate oak from Serbia.

MATERIALS AND METHODS

Sampling Site and Procedures

Sampling was conducted in a 18-year-old pedun-culate oak (*Quercus robur* L.) stand (N 44°56'55.5", E 19°10'56.7" and 82 m a.s .l.) situated in forest administration "Morović" under the management of Public Enterprise "Vojvodinašume". Also, *Acer campestre* L., *Cornus sanguinea* L. and *Ulmus effusa* Willd were present with a minor share.

In the meteorological station in Sremska Mitrovica the mean annual temperature for the period 1981-2010 of 11.2°C and the average annual amount of precipitation of 641.64 mm were recorded. Based on the average monthly sum of precipitation in this area, June was the month with the most precipitation, while February was the driest (RHMZ 2021).

Soil sampling was performed at the beginning of July 2018. Six soil samples were taken with a standardized soil corer (Kraigher 1999) with total volume of 274 ml and length of 18 cm. Soil samples were stored in the fridge for up to three months. To loosen the soil structure, each sample was submerged overnight in tap water before analyses. All fine roots were carefully washed from the soil and divided into vital ECM root tips or old, non-turgescent and non-mycorrhizal roots using a dissecting microscope Olympus SZX 10 (Olympus Corp., Tokyo Japan) with magnifications 10-63× (light source: Olympus Highlight 3100, daylight filter). Vital ECM root tips were classified into different morphotypes of ectomycorrhizae based on their morphological and anatomical characteristics, using a dissecting microscope and a microscope (Olympus BX 53®, Olympus Corp., Tokyo Japan) with magnifications 100-1000×. Morphotypes of ectomycorrhizae were described according to the methodology proposed by Agerer (1991) and Kraigher (1996). If it was possible, a fungus from ectomycorrhiza was identified by comparison with descriptions published in Agerer et al. (2006), Agerer (2008), or Agerer and Rambold (2021).

Morphotypes of ectomycorrhizae were categorized into the exploration types as suggested by Agerer (2001). All categories of fine root tips were quantified by counting under the dissecting microscope.

Molecular Identification of Ectomycorrhizal Fungi

Molecular identification of fungus in ectomycorrhiza was based on PCR amplification of internal transcribed spacer (ITS) region of fungal nuclear rDNA. DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from ECM root tips. DNA extraction of some morphotypes of ectomycorrhiza was unsuccessful and morpho-anatomical identification was not sufficient to determine the ECM fungus. In that case, ECM morphotype was labeled as an "unidentified" type. Amplification reactions were performed using ITS 1F (Gardes and Bruns 1993) and ITS 4 primer pair (White et al. 1990) in Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). The content of PCR mixture and thermal cycling conditions were explained by Milović et al. (2021b). Amplified DNA fragments were separated and purified from the agarose gel using the QIAquick gel extraction kit and QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and then sent for sequencing to Macrogen Europe B.V. The determination of species, genus, or family of ECM fungi was done by comparison of the obtained sequences to those deposited in GenBank (NCBI 2021) and UNITE (Nilsson et al. 2018) databases.

Data Analysis

Calculation of diversity indexes was performed per sample and per site (i.e., by pooling the data of ECM community) following the formulas given by Atlas and Bartha (1981) and Taylor et al. (2000), which was explained in more detail in the paper by Milović et al. (2021b).

RESULTS AND DISCUSSION

Eighteen ECM fungal taxa were recorded in the young pedunculate oak stand from Morović, among which seven were determined to the level of species, eight to the genus, two to the level of family and one morphotype remained unidentified (Figure 1).

An analysis of ectomycorrhizas in oak plots across European countries, based on 96 soil cores taken per plot, during one season showed that the average richness was 55, ranging from 24 fungal taxa in the Netherlands to 83 in Romania (Suz et al. 2014). A study of temporal changes in the ECM community of a temperate oak forest in northeast France revealed 75 fungal taxa within 15 months (6 soil cores were taken each month) (Courty et al. 2008). Investigating the diversity of ECM morphotypes and tree decline in two stands of pedunculate and sessile oaks in Austria, Kovacs et al. (2000) recorded 46 and 38 ECM morphotypes within 3 years in a total of 216 soil samples per site. On the other hand, the number of taxa obtained in our research was similar to the ones recorded in two sessile oak stands from Fruška Gora where 17 and 12 ECM taxa were determined in ten soil samples per site (Milović et al. 2021b). Furthermore, in three declining pedunculate oak stands in western Poland the number of ECM fungal taxa per site ranged from 11 to 15 (based on 60 soil samples
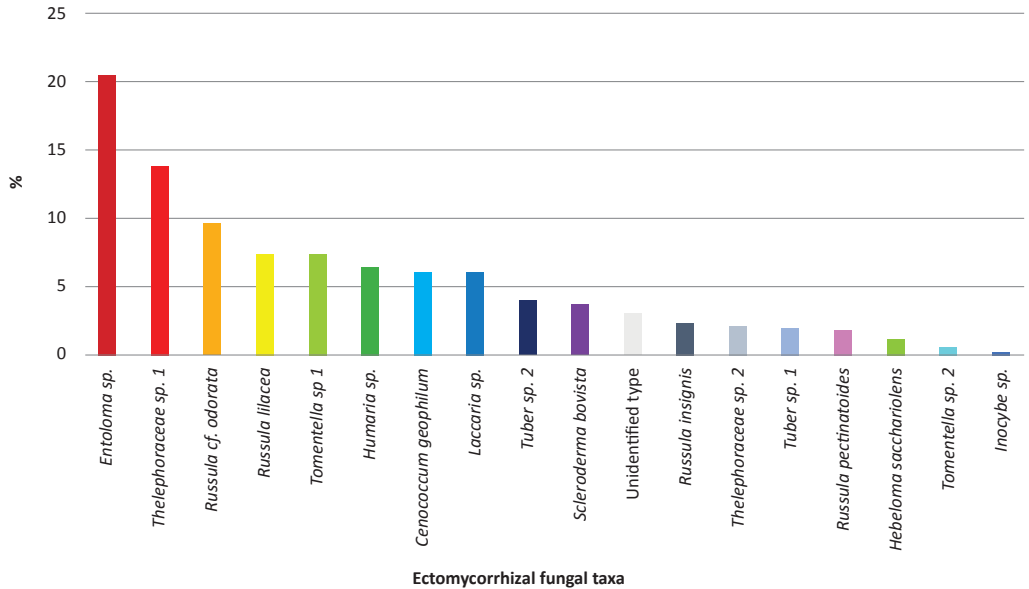
Figure 1. Relative abundance of ectomycorrhizal fungal taxa (based on the number of ectomycorrhizal root tips belonging to a particular ectomycorrhizal fungal taxon in relation to all analysed ectomycorrhizal root tips) in a young pedunculate oak stand from the Morović site.

taken per site) (Bzdyk et al. 2019). Similarly, investigating the effect of dead wood on ECM colonization in an old growth pedunculate oak forest in north-eastern Poland, Olchowik et al. (2019) recorded fungal species richness in the range from 12 to 14 taxa in 20 soil samples per site. On sessile oak from the Taurus mountains in Central Germany, 18 ECM fungal taxa were recorded (Schirkonyer et al. 2013), while on native oaks (pedunculate and sessile oak) from Ireland 21 ECM taxa were determined in the total of 16 soil samples (O'Hanlon and Harrington 2012). However, the average species richness across three pedunculate oak-dominated woodlands in southern England was 33 and it was in the range from 17 to 45 (Barsoum et al. 2021) (based on 80 soil cores per woodland).

On average, six ECM fungal taxa were recorded in soil samples from the Morović site (Table 1). This is a bit lower in comparison to vital oak trees in Austria (Kovacs et al. 2000) and control stand in *Q. ilex* forest from Spain (de Roman and de Miguel 2005) where on average nine and seven morphotypes were found, respectively. On the other hand, in soil samples from two sessile oak stands from Fruška Gora, only three ECM fungal taxa were found on average (Milović et al. 2021b).

At the Morović site, Shannon-Weaver index with value of 1.4 per soil sample (Table 1) is similar to the one noted in oaks stands in Austria where vital trees had this index in the range 1.3-1.5 (Kovacs et al. 2000) and control stand of *Q. ilex* in Spain where it was 1.3 (de Roman and de Miguel 2005). On

Table 1. Total and average values of the number of ectomycorrhizal fungal taxa, the number of vital ectomycorrhizal root tips, the number of old, non-turgescent and non-mycorrhizal root tips, % of vital ectomycorrhizal root tips and diversity indices in young pedunculate oak (*Quercus petrea* L.) stand located at the Morović site.

Parameter	Site	Sample
	Total	Average±SE
Number of ectomycorrhizal fungal taxa	18	5.6±0.4
Number of vital ectomycorrhizal root tips	2145	342.4±50.92
Number of old, non-turgescent and non-mycorrhizal root tips	8996	1507.2±406.9
% of vital ectomycorrhizal root tips	19.2	22.5±6
Species richness index	5.10	1.83±0.21
Shannon-Weaver index	2.54	1.44±0.10
Evenness	2.02	1.71±0.08
Equitability	0.88	1.94±0.11
Berger-Parker index	0.79	0.61±0.05

the other hand, the obtained value is higher than in sessile oak stands from Fruška Gora where Shannon-Weaver index was 0.7 and 0.9 (Milović et al. 2021b). Total value of Shannon-Weaver index calculated for site Morović (2.5) is comparable to the one recorded by Barsoum et al. (2021) in pedunculate oak woodlands from southern Britain, which ranged from 1.9 to 2.8. Similar values were noted in Austria for aggregated soil samples taken under vital trees which varied from 2.6 to 2.7 (Kovacs et al. 2000) and in two sessile oak stands from Fruška Gora (2.1 and 2.3) (Milović et al. 2021b).

The most abundant ECM fungi at the studied site were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea*, which together associated with more than a half of all ECM root tips (Figure 1). ECM community was made up of a small number of abundant fungal taxa and a bigger number of rare taxa, which is in accordance with numerous studies (Suz et al. 2014, Rosinger et al. 2018, Bzdyk et al. 2019, Milović et al. 2021b, Barsoum et al. 2021). According to Courty et al. (2008), abundant ECM fungi are usually present all year long, some species occur all year long with fluctuating abundance, while some ECM fungi are characteristic for a particular season or month. Occurrence of a large number of taxa present at low frequencies is important for the stability of the forest stand. If ecological conditions change, some rare ECM species might be adapted to new conditions, while some dominant ECM fungi might not (Pena et al. 2010, O'Hanlon and Harrington 2012).

At the studied site, ECM fungi *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata*, *Russula lilacea* and *Tomentella* sp. 1 made associations with the majority of ECM root tips (Figure 1). Their abundances were in the following range: 7-21%. In temperate oak plots across nine European countries, the most abundant ECM fungal species were *Lactarius quietus*, *Cenococcum geophilum,* and *Russula ochroleuca* (Suz et al. 2014). Moreover, *Lactarius quietus*, *Tomentella sublilacina*, *Cenococcum geophilum*, and *Russula* sp. 1 were abundant in a temperate oak forest in northeastern France and present all year long (Courty et al. 2008). The most abundant ECM species in pedunculate oak woodlands in southern England were *Lactarius quietus*, *Cenococcum geophilum*, *Boletus rubellus*, *Tomentella sublilacina* and *Lactarius subdulcis* (Barsoum et al. 2021). *Lactarius quietus, Cenococcum geophilum,* and *Tomentella sublilacina* were also found in two sessile oak stands on Fruška Gora (Milović et al. 2021b). *Lactarius quietus* is an oak specialist and one of the most abundant and frequently occurring ECM fungi in temperate oak forest across Europe (Voříšková et al. 2013, Bzdyk et al. 2019). It is interesting to notice that *Lactarius quietus* was not recorded in the studied young pedunculate oak stand at the Morović site as well as any other ECM fungal taxon from genus *Lactarius*. ECM type identified as *Cenococcum geophilum* made association with 6% of all ECM root tips. *Cenococcum geophilum* is regarded as the most widely distributed species complex that makes symbiosis with over 200 tree species (LoBuglio 1999). It has an ability to resist extreme conditions (drought especially), and it is frequently observed on oak trees in Europe (de Roman and Miguel 2005, Schirkonyer et al. 2013, Milović et al. 2021b).

Thelephoraceae, *Russulaceae*, and *Entolomataceae* were dominant fungal taxonomic families at the Morović site, which colonized more than 65% of all vital ECM root tips (Figure 2). Moreover, families *Thelephoraceae*

Figure 2. Relative abundance of taxonomic families of ectomycorrhizal fungi based on the number of ectomycorrhizal root tips belonging to a particular family in relation to all analyzed ectomycorrhizal root tips in young pedunculate oak stand from the Morović site.
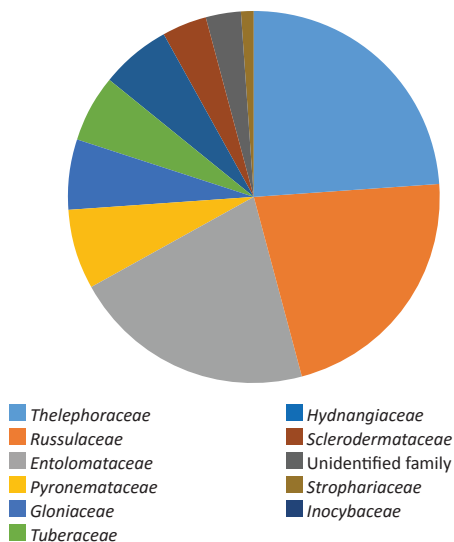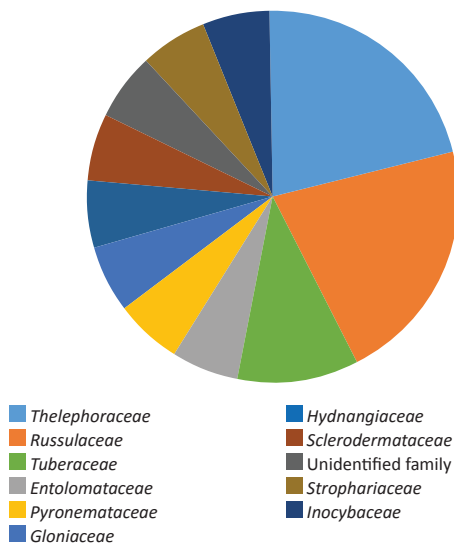

Figure 3. Species richness of taxonomic families of ectomycorrhizal fungi based on the number of ectomycorrhizal taxa belonging to a particular family in relation to the number of all determined ectomycorrhizal taxa in young pedunculate oak stand from the Morović site.

and *Russulaceae*, each containing four taxa, were the most species-rich families at this site (Figure 3). Families *Russulaceae, Cortinariaceae* and *Thelephoraceae* were noted as dominant in temperate and Mediterranean oak forests (Courty et al. 2008, Richard et al. 2011). In two sessile oak stands from Fruška Gora dominant fungal families were *Thelephoraceae*, *Russulaceae*, and *Inocybaceae* at the Infocenter site and *Entolomataceae*, *Tuberaceae*, *Russulaceae* at the Brankovac site (Milović et al. 2021b). Furthermore, the study of ECM diversity in temperate oak forests throughout Europe reported that the most abundant families were *Russulaceae*, *Gloniaceae*, *Thelephoraceae*, and *Cortinariaceae* (Suz et al. 2014). Similarly, in pedunculate oak woodlands from south England *Russulaceae*, *Boletaceae*, *Gloniaceae* and *Thelephoraceae* were dominant (Barsoum et al. 2021).

Higher number of ECM fungi from the phylum Basidiomycota in comparison to Ascomycota obtained in our study (Table 2) is in accordance with numerous earlier studies (Richard et al. 2011, Suz et al. 2014, Bzdyk et al. 2019, Milović et al. 2021b etc.).
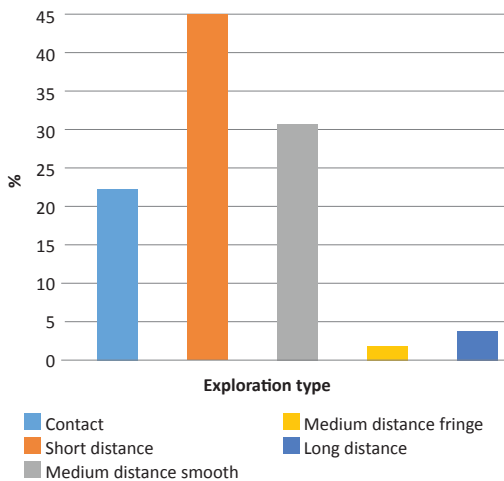
According to Agerer (2001), classification of ectomycorrhizae on ETs links fungal morphology (especially the amount and differentiation of extraradical mycelium) with their ecology. Different ETs have different functional characteristics and different capacities for taking up and translocation of nutrients and carbon storage (Hobbie and Agerer 2010). Moreover, ETs can be used for the purpose of site indication, primarily to indicate the composition of soil and the availability of nutrients (Rudawska et al. 2011).

In the studied young pedunculate oak from the Morović site, short-distance ET dominated, followed by medium-distance smooth ET and contact ET, while long-distance ET and medium-distance fringe ET were rare (Figure 4). Mycorrhizae with contact, short- and medium-distance smooth ETs are considered to use labile, mainly inorganic nitrogen (N), while medium distance fringe and mat, and long-distance ETs are assumed to use organic N (Lilleskova et al. 2011, Suz et al. 2014). Furthermore, the long-distance ET, with abundant mycelial biomass was found to be well adapted to nutrient-deficiently conditions, being especially deficient in N (Hobbie and Agerer 2010). Investigating ETs of ECM fungi in declining oak stands, Bzdyk et al. (2019) noted that the abundance of contact ET was positively correlated with C:N ratio and organic carbon content, while the abundance of short distance ET was closely related to calcium and phosphorus (P_2O_5) content and pH.

Contact or short-distance ETs of ECM fungi are assumed to mostly have wide environmental ranges (Rosinger et al. 2018). Shorter distance ETs are characteristic for colder climates where soils are well provided with total N and plants invest less carbon in mycorrhizal association (Rosinger et al. 2018). On the other hand, in warmer and less fertile environments dominated long-distance ETs of ECM fungi which demand more carbon (Rosinger et al. 2018, Defrenne et al. 2019). A high abundance of shorter distance ETs (contact, short- and medium-distance smooth ETs) recorded in analyzed young pedunculate oak stand from the Morović site suggests that this soil is sufficiently provided with total nitrogen and organic carbon and that therefore nutrients can be reached without bigger carbon investments in extraradical mycelium. Similar results were obtained in sessile oak stands from Fruška Gora (Milović et al. 2021b).

Bzdyk et al. (2019) found the connection between the diversity of ETs and the tree health status, which could indicate potential positive role of ECM functional diversity. Also, a high diversity of ETs could make ECM fungal communities more resilient to environmental change (Suz et al. 2014).

Figure 4. Relative abundance (%) of ectomycorrhizal exploration types (based on the number of ectomycorrhizal root tips belonging to a particular exploration type in relation to all analyzed ectomycorrhizal root tips) in young pedunculate oak stand from the Morović site.

Table 2. The number of ectomycorrhizal root tips and ectomycorrhizal fungal taxa belonging to Ascomycota and Basidiomycota in young pedunculate oak stand from the Morović site.

	Ascomycota	Basidiomycota
	N	
Ectomycorrhizal root tips	409	1736
Ectomycorrhizal fungal taxa	4	14

CONCLUSIONS

This paper presents the first underground insight into the diversity of ECM fungi on pedunculate oak in Serbia. In the studied young pedunculate oak stand from Morović, 18 ECM fungal taxa were recorded, described and identified mostly with molecular methods. The most abundant ECM fungi were *Entoloma* sp., *Thelephoraceae* sp., *Russula* cf. *odorata* and *Russula lilacea* while short-distance exploration type ET dominated, followed by medium-distance smooth ET and contact ET. Values of diversity indices of ECM fungi in the studied pedunculate oak stand were comparable to European research on the ECM diversity in oaks. The obtained results indicate the need to continue research on increased number of sites and to include seasonal dynamics to get a deeper insight into the diversity of ECM fungi on pedunculate oak.

Author Contributions

MM and SO conceived and designed the research, MM and LK carried out the field measurements, MM performed laboratory analysis, MM and BK processed the data and performed the statistical analysis, SO secured the research funding and supervised the research, SP, AP and BK helped to draft the manuscript, MM, BK and AGM wrote the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Supplementary Materials

Supplementary File 1 - Ectomycorrhizal fungi recorded in the young pedunculate oak stand from the Morović site identified on the basis of morpho-anatomical characteristics and molecular methods (based on the similarity with the sequences from publicly available nucleotide databases (GenBank and UNITE) and their absolute / relative (%) abundance. Sequences were last compared to nucleotide databases on 15th September 2021.

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ORIGINAL SCIENTIFIC PAPER

Acorn Yields and Seed Viability of Pedunculate Oak in a 10-year Period in Forest Seed Objects across Croatia

Anđelina Gavranović Markić[1,*], Saša Bogdan[2], Marija Gradečki Poštenjak[3], Miran Lanšćak[1], Zvonimir Vujnović[1], Sanja Bogunović[1], Mladen Ivanković[1,*]

(1) Croatian Forest Research Institute, Division of Genetics, Forest Tree Breeding and Seed Science, Cvjetno naselje 41, HR-10450 Jastrebarsko, Croatia; (2) University of Zagreb, Faculty of Forestry and Wood Technology, Department of Forest Genetics, Dendrology and Botany, Svetošimunska 25, HR-10000 Zagreb, Croatia; (3) Croatian Forest Research Institute, Division for Silviculture, Cvjetno naselje 41, HR-10450 Jastrebarsko, Croatia

* Correspondence: e-mail: andelina@sumins.hr; mladeni@sumins.hr

ABSTRACT

Quercus robur L. exhibits not only a large inter-annual variability in seed production but also considerable variability among locations and individuals within the same year. The differences in how individual trees and populations of oaks invest in acorn production, both in terms of the yield size and the acorns quality, is of interest both ecologically and economically. For this research we used data collected from harvesting which has been organised and executed by the largest forestry company in Croatia - public enterprise Croatian Forests Ltd. According to the Act on Forest Reproductive Material, Croatian Forest Research Institute is designated as the Official Body that supervises production and provides analysis of quality of forest reproductive material. Regarding that, we summarized data of 10-year long records (from 2009 to 2018) of submitted seed samples and seed quality testing from 119 *Q. robur* forest seed objects across Croatia. Our aim was to investigate seed yield and effects of seed size and seed moisture content on seed viability. In the study period there were four years with higher quantity of collected seeds than other years (2010, 2011, 2015 and 2017). There was no significant difference in seed viability among forest seed objects. However, variations among years within forest seed objects were highly significant. It was also found that seed size (indicated by the number of acorns per kg) and seed moisture content were significantly related to seed viability. Bigger seed dimensions, i.e. lower mean number of acorns per kg, correlated with higher viability. Lower moisture content affected decrease in acorn viability. On average, viability dropped below 70% when acorn mean moisture decreased below 36%. Thus, it can be concluded that bigger seeds and seeds with moisture content of 40-44% have better viability.

Keywords: *Quercus robur* L.; acorn crop; moisture content; seed size

INTRODUCTION

There is a growing interest in understanding (and being able to predict) seed yield, an essential prerequisite for successful stand regeneration and its further development. Since acorn production is a foundational process of ecosystems dominated by oaks, it is critical to the sustainable management of oak forests. For oaks, a defining characteristic are spatial-temporal variations in large synchronous but intermittent seed yields (Liebhold et al. 2004, Bogdziewicz et al. 2018). Synchrony is the extent to which seed crops are correlated across years, while temporal variability is the among-year variation in seed yield (Liebhold et al. 2004). Such synchronous seed production, which fluctuates strongly from year to year, is called masting and has been frequently documented in oak species (Kelly 1994, Greenberg and Parresol 2002).

The pedunculate oak begins fructification at the age of 40–50 years in the open and at the age of 70–80 years in dense canopies (Martiník et al. 2014). In general, a large acorn crop is expected every 6-9 years and a moderate crop every 3-4 years (Worrel and Nixon 1991, Harmer 1994). In

any particular year, seed production varies among stands (Crawley and Long 1995, den Ouden et al. 2004, Kasprzyk et al. 2014, Pesendorfer et al. 2020). Even in poor crop years individual trees and stands will produce some seeds. Similarly, in good crop years, certain stands may fail to produce seeds (Harmer 1994, Wesołowski 2015). In addition to the high variation in seed production among years, there are also considerable variations among individual oaks within the same year (Caignard et al. 2019). The cause of periodic seed production is unclear, but likely related to an interaction between environmental, endogenous and biological factors over a longer period.

Besides quantity, a basic prerequisite for successful natural and artificial regeneration is the quality of seed (Poštenjak and Gradečki 2001, Gradečki-Poštenjak et al. 2018). It is possible to make visual assessment of acorn yield in August/September (Van der Meer et al. 2002), but the quality of seed crop cannot be known until seed dispersal in autumn. Quality of acorns is determined by many factors, including size, weight, moisture content, mineral nutrient reserves, and the percentage of seeds that germinate or are capable of germinating (seed viability) (Poštenjak and Gradečki 2001, Nikolić et al. 2006, Devetaković et al. 2019). A combination of environmental factors during seed development and the genetic variability can result in variations in seed dimensions (Willan 1985). Larger seed dimensions indicate better seed quality, germination and genetic potential (Toon et al. 1990, Davidson et al. 1996), but seed quality may depend on several factors, such as the time of the harvest (Bellari and Tani 1993), variation in the nutrient content (Abideen et al. 1993) and genetic factors (Farmer 1980, Jayasankar et al. 1999). After falling to the ground, the seeds should be promptly collected and cleaned. Prolonged time on the ground and suboptimal cleaning may adversely affect seed viability. Generally, the lower the quality of the seeds, the faster they deteriorate. After harvesting, acorns have a moisture content ranging from 40% (Suszka et al. 2000, Szabla and Pabian 2009) to 50% (Aniszewska et al. 2020). Fresh acorns are specific because of high moisture content, and they do not tolerate moisture loss without adverse effect on its viability (Doody and O'Reilly 2008). The most relevant method for testing seed quality in practical forestry is testing the germination capacity of seeds (Bonner 1974a), but this method takes several weeks and for some species pre-treatment may take some additional weeks or months. On the other hand, the viability test, i.e. the topographical tetrazolium test, is a method that also allows the viability of seeds to be assessed accurately, but much more quickly than with the germination test. The viability test plays an important role in assessing the physiological quality of the seeds and the percentage of viability, and it provides valuable information for diagnosing possible problems with seed quality, such as mechanical damage and insect damage.

In the literature, mast years are referred to as years when acorns are produced in substantial quantities (Isagi et al. 1997, Sever et al. 2013). Greenberg and Parresol (2002) defined mast year as the year of production higher than the 5-year average production. In Croatia, the main problem is that there has not been long-term monitoring of the yield of forest trees, and it is not possible to precisely determine the actual production potential of individual forest stands. The current practice is that forest offices (FOs) and/or forest administrations (FAs) stop collecting seeds when they ensure and cover their own needs. To our knowledge, long-term monitoring of pedunculate oak crop in Croatia was conducted from 1976 to 1995 on a pedunculate oak stand in Lipovljani (Matić et al. 1996a), from 1968 to 1994 in a larger area of FA Vinkovci (Matić et al. 1996b), and from 2000 to 2016 in the Spačva Basin area (Gradečki-Poštenjak et al. 2011, Gradečki-Poštenjak 2017).

For this paper, we used data of a 10-year-long record of annual acorn yield (2009-2018) from 119 pedunculate oak forest seed objects (FSOs) across Croatia. Our objectives were to investigate (1) the quantity of pedunculate oak acorns (i.e. the number of seed samples) during 10 years in Croatia and to determine (2) the relationship between seed viability and moisture content and (3) the relationship between seed viability and seed size. To our knowledge, this is the first attempt to summarize the data on monitoring of the yield of one of the most important forest tree species in Croatia.

MATERIALS AND METHODS

Acorn crops were collected from 2009 to 2018 in 119 pedunculate oak FSOs (seed sources, seed stands) across Croatia. We used data collected from harvesting which has been organised and executed by the largest forestry company in Croatia – public enterprise Croatian Forests Ltd. Control of seed collection, sampling and quality testing was carried out by the Croatian Forest Research Institute (CFRI) – the Official Body according to the Act on Forest Reproductive Material (Official Gazette 75/09, 61/11, 56/13, 14/14, 32/19, 98/19). Sampling procedures and seed quality testing have been performed by the Laboratory for Seed Testing (LIS) of the CFRI according to International Rules for Seed Testing (ISTA Rules).

Sampling Procedures

A seed lot (i.e. acorn crop harvested from the same FSO in a season) quality has been determined by analysing seed samples, which were assumed as representative parts of a crop. The seed lots were stored in such a way that all its parts were accessible for sampling, separated from other seed lots and marked so they could be easily identified. Issued lot and sample sizes for *Quercus* sp. are given in Table 1. The composite sample was formed by combining and mixing primary samples taken from different positions of a seed lot and subsamples, which were combined to create the submitted sample for moisture content. Samples were sealed and labelled according to the following data: species, register number of forest seed object, number of the package/total number of packages constituting the composite sample, total weight of the seed lot, and weight of the seed sample or the number of seeds in the sample. The submitted samples were transferred to the LIS as soon as possible.

Table 1. ISTA Rules: Table 2C Part 2 – Lot sizes and sample sizes: Tree and shrub species (part) (ISTA).

Species	Maximum weight of lot (kg)	Minimum submitted sample (pcs)	Minimum working sample for purity analyses (pcs)
Quercus spp.	5000	500	500

The Laboratory Procedure

The following analyses were conducted on all seed samples: purity analysis, thousand-seed weight (TSW) determination, moisture content (MC), and topographical tetrazolium test for seed viability (SV). The objective of the purity analysis was to determine weight proportion of pure seeds in a sample (pedunculate oak acorns without any other seeds or matters like twigs, leaves etc.). It was the first analysis to be carried out and subsequent analyses were made on the pure seed component of the sample. The working sample for TSW determination consisted of 400 pure seeds taken randomly and determined by mean of eight replicates, each containing 50 seeds. The MC was measured according to the ISTA low-temperature oven method. Seeds were dried in a forced ventilated oven at 103 °C for 17 h. The difference between a fresh sample weight and its oven-dried weight divided by the fresh weight was recorded as the sample moisture content proportion. Acorn SV was examined with the tetrazolium method. The seeds were soaked in 2,3,5-Triphenyl tetrazolium chloride, which is an indicator of cellular respiration. Viable seeds were stained while non-viable seeds remained white. The proportion of stained acorns in a sample was a sign of its viability.

In order to calculate the number of acorns per kg ($N \cdot kg^{-1}$) in the original seed lot from which the submitted sample was taken, the following formula was applied (Forestry Commission 2018):

N of seeds per kg ($N \cdot kg^{-1}$) = 1000/T x 1000 x P/1000

where T is 1000 pure seed weight in grams and P is purity % from the purity test.

Finally, data on seed sample moisture content (MC %), seed sample viability (SV %) and number of acorns·kg^{-1} per sample ($N \cdot kg^{-1}$ – indicating acorn size) were further processed.

Data Processing and Statistical Analysis

In the 10-year period the total amount of 2,732 seed samples taken from 119 pedunculate oak FSOs were analysed by the LIS. However, data from the years in which the greatest number of FOs submitted seed samples were taken into consideration for this study. The period of 2009-2018 included: year 2010 (when 35 FOs submitted seed samples), year 2011 (32 FOs), year 2015 (29 FOs) and year 2017 (27 FOs). Furthermore, only data from FOs represented by at least two seed samples from a single FSO in all years with higher number of seed samples were additionally filtered. In such a way, data of 15 FOs with various numbers of seed samples per FSO (at least two) and from the four years were further processed.

Statistical analyses were conducted using RStudio ver. 1.2.5001 (RStudio Team 2019) and SAS/STAT 15.1 software

(SAS Institute Inc. 2018). Descriptive analysis was performed using the MEANS procedure in SAS to calculate arithmetic means and standard deviations of the traits. Analysis of variance (ANOVA) was performed using the MIXED procedure in SAS, to establish statistical significance of analysed factors for the SV trait. Fixed effect of a seed stand *i* (*i*=1….15), random effect of a higher sample number years *y* (*y*=1…4), random effect of MC and random effect of acorn size ($N \cdot kg^{-1}$, being average number of acorns per kg) were the analysed factors. Pearson's correlation analyses were performed using the Hmisc package in R (Harrell et al. 2019) to determine significant associations between the mean SV and other traits (MC and $N \cdot kg^{-1}$). The correlation analyses were done with data combined for all seed stands as well as with a single stand data.

RESULTS

Data on MC, $N \cdot kg^{-1}$ and SV from 15 FOs were analysed. In them, FSOs were represented by at least 2 seed samples in each of the four higher sample number years (Table 2). The highest average estimates of MC were in 2015 and 2017 (MC = 41.1%). In 2015, MC ranged from 43.8% in FOs Otok and Gunja to 36.8% in FO Lekenik, while in 2017, it ranged from 44.1% in FO Nova Gradiška to 37.6% in FO Kutina. The lowest average MC was in 2011 (35.8%), ranging from a minimum of 33.1% (FO Vrbanja) to a maximum of 38.5% (FO Garešnica). The largest average $N \cdot kg^{-1}$ was in 2017 (189 acorns·kg^{-1}), ranging from 221 acorns·kg^{-1} in FO Valpovo to 160 acorns·kg^{-1} in FO Nova Gradiška. In 2015, there were the smallest $N \cdot kg^{-1}$ (172 acorns·kg^{-1}), ranging from 204 acorns·kg^{-1} in FO Sisak to 137 acorns·kg^{-1} in FO Kutina. On average, SV was the most consistent in 2010, when it ranged from a minimum of 74.3% in FO Nova Gradiška to a maximum of 88% in FO Daruvar. In 2011, SV was the most inconsistent, when it ranged from a minimum of 38.5% in FO Vrbanja to a maximum of 92.7% in FO Garešnica.

In general, there were no significant differences in SV among FOs or FSOs, i.e. the stand effect was not significant for this trait (Table 3). However, the years-within-stand effect was statistically significant indicating differences in the SV within FSOs across various years. Likewise, the seed-moisture effect and the acorn-size effect (both nested within a stand) were significant as well (Table 3).

SV from FSOs greatly varied among the years with higher sample number and these variations were observed in samples from all FSOs (Figure 1, showing these variations in just two FSOs).

Correlation analyses revealed significant positive relationship between average SV and average MC (when combined all data; R=0.32, p<0.0001), but this correlation was even more pronounced when data were analysed separately by FSOs (Figure 2).

Table 2. Basic descriptive statistics of studied acorn crop traits from selected FSOs in FOs across various years.

Forest office (FO)	Number of samples	MC (%)	SD	N·kg⁻¹	SD	SV (%)	SD
Year 2010							
Bjelovar	3	39.7	0.8	188	7.3	79.7	0.6
Daruvar	2	38.9	0.1	165	11.7	88.0	7.1
Donji Miholjac	5	39.6	2.0	183	18.7	84.6	8.3
Garešnica	2	40.3	0.5	159	19.9	82.5	2.1
Gunja	144	41.3	2.1	191	17.5	75.9	6.3
Koprivnica	5	37.0	1.8	186	17.3	78.6	5.6
Kutina	12	39.6	1.8	193	9.0	84.3	3.8
Lekenik	5	40.7	1.2	185	11.8	77.2	4.1
Nova Gradiška	13	40.1	4.5	185	18.5	74.3	5.3
Otok	27	40.7	2.6	181	24.3	83.7	4.9
Sisak	14	39.6	1.8	173	12.5	85.2	6.8
Slatina	3	41.6	0.7	172	18.7	83.3	13.4
Sunja	3	43.5	3.7	149	20.6	86.3	2.5
Valpovo	2	39.1	0.9	205	5.2	81.5	0.7
Vrbanja	32	41.6	1.8	194	30.8	83.9	7.5
Year 2011							
Bjelovar	9	35.9	2.2	211	12.7	62.8	6.8
Daruvar	2	35.6	2.2	179	2.6	82.0	0.0
Donji Miholjac	7	37.5	1.4	173	6.4	73.9	4.6
Garešnica	3	38.5	1.8	137	6.4	92.7	1.2
Gunja	101	36.3	1.6	203	24.6	74.5	8.2
Koprivnica	5	35.2	2.1	193	18.9	69.8	8.0
Kutina	4	35.1	4.5	153	12.2	75.3	5.4
Lekenik	3	36.8	1.3	143	19.1	73.7	3.5
Nova Gradiška	3	35.9	3.0	182	25.0	46.0	21.1
Otok	6	34.2	1.9	214	18.9	58.8	11.9
Sisak	6	35.2	1.5	150	16.1	72.0	4.6
Slatina	6	35.4	0.8	183	25.1	72.0	4.0
Sunja	5	37.6	2.4	133	22.0	72.6	3.2
Valpovo	9	35.3	0.9	173	22.6	66.6	6.3
Vrbanja	19	33.1	2.0	214	16.5	38.5	5.3
Year 2015							
Bjelovar	19	41.6	1.9	159	14.4	78.9	7.1
Daruvar	2	40.2	1.2	168	5.1	80.3	1.8
Donji Miholjac	33	42.4	2.4	174	10.7	74.3	5.3
Garešnica	8	42.8	2.5	185	28.0	82.6	6.2
Gunja	21	43.8	2.3	171	5.1	76.3	4.4
Koprivnica	4	39.8	3.4	165	13.0	79.0	7.7
Kutina	17	41.0	1.8	137	21.5	83.1	5.3
Lekenik	9	36.8	2.6	190	15.3	59.6	13.9
Nova Gradiška	3	40.1	0.3	163	10.4	74.0	1.3
Otok	7	43.8	1.2	172	6.2	76.1	4.9
Sisak	6	37.2	2.7	204	32.7	70.3	6.3
Slatina	6	42.5	2.0	170	13.1	79.2	6.2
Sunja	24	41.7	1.6	174	27.2	83.0	6.3
Valpovo	5	41.6	2.3	170	4.1	74.9	2.0
Vrbanja	23	41.9	1.4	174	9.2	76.3	6.0
Year 2017							
Bjelovar	6	40.3	1.2	174	12.6	82.2	2.6
Daruvar	5	40.2	1.6	184	16.9	86.3	2.6
Donji Miholjac	2	41.5	1.3	197	0.2	79.3	5.3
Garešnica	10	41.1	3.0	172	8.2	79.6	5.3
Gunja	8	42.4	1.5	200	16.6	81.7	9.4
Koprivnica	16	39.0	3.2	194	11.6	70.7	9.4
Kutina	2	37.6	0.0	173	1.7	85.5	0.0
Lekenik	13	40.3	1.3	179	14.0	72.2	7.9
Nova Gradiška	2	44.1	1.5	160	6.1	50.5	4.2
Otok	16	42.2	1.8	195	9.9	73.9	10.9
Sisak	12	40.7	1.6	185	6.3	59.4	5.7
Slatina	35	42.0	2.9	186	12.9	72.3	7.9
Sunja	5	40.8	1.7	211	4.9	61.8	7.8
Valpovo	6	41.1	2.4	221	16.9	86.3	1.8
Vrbanja	13	42.9	4.3	209	18.6	79.6	7.2

MC – mean seed moisture content; SD – standard deviation; N·kg⁻¹ – average number of acorns per kg; SV – mean seed viability

Table 3. Factorial ANOVA summary for viability of *Q. robur* acorns (SV). Acorns from selected FSOs in 15 FOs were sampled in four mast years. Analysed factors were: fixed effect of a stand (levels: 1....15), random nested effects of: years-within-stand [Years(Stand)], acorn moisture-within-stand [Moist(Stand)] and acorn size-within-stand [Size(Stand)]. Significance of the factors is indicated by red colour (α=0.05).

Fixed effects	F	Pr>(F)
Stand (FO)	1.55	0.1058
Random effects	**Z**	**Pr>(Z)**
Years(Stand)	4.52	<.0001
Moist(Stand)	1.98	0.0241
Size(Stand)	2.11	0.0174

Therefore, decreased SV in 2010, 2011 and 2015 may be explained by lower MC, at least partially. For example, FO Lekenik in 2015 had the lowest mean MC (36.8%) and low SV (59.6%). In the same year, the FO Gunja with the highest MC (43.8%) had highest SV, 76.3% (see Table 2).

Significant negative correlation was identified between average $N \cdot kg^{-1}$ and mean SV (when all data are combined, R=-0.22; p<0.0001). This relationship was even more pronounced when data were analysed separately by FSOs as well (Figure 3). In fact, this relationship revealed positive correlation between mean SV and its average size, because higher mean $N \cdot kg^{-1}$ means smaller average acorns size. For example, in 2015, there were on average 137 acorns per kg and 83.1% viable acorns from FO Kutina. On the contrary, FO Sisak was featured with a mean of 204 acorns per kg and 70.3% viable seeds (see Table 2).
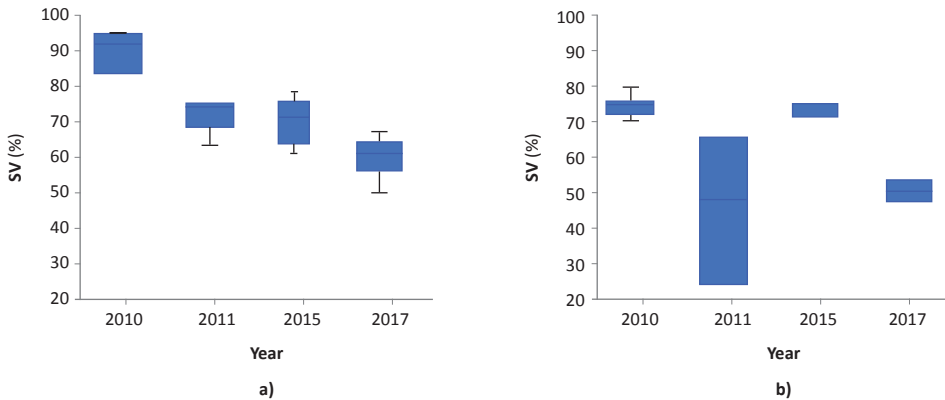

Figure 1. SV distributions across the higher sample number years: **(a)** acorns from the FSO in Sisak and **(b)** acorns from the FSO in Nova Gradiška. The graphs show large variations in SV from the same FSO among years.
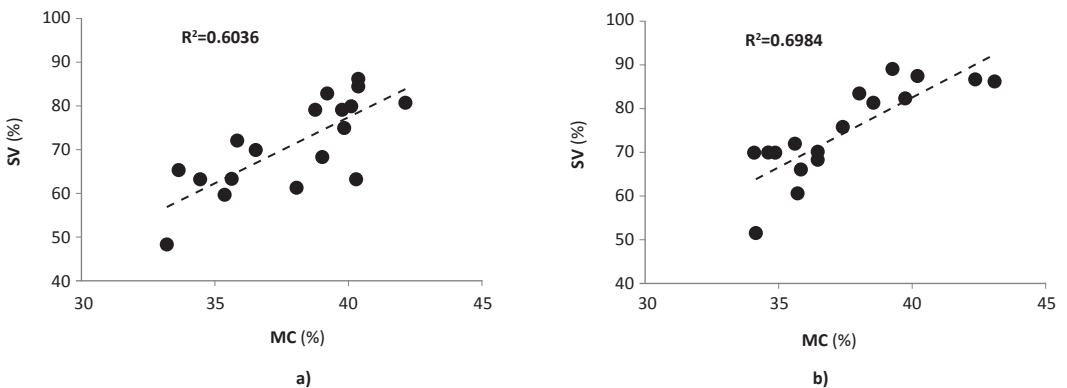

Figure 2. Correlation between mean SV and mean MC: **(a)** data from the FSO in Bjelovar and **(b)** data from the FSO in Valpovo.
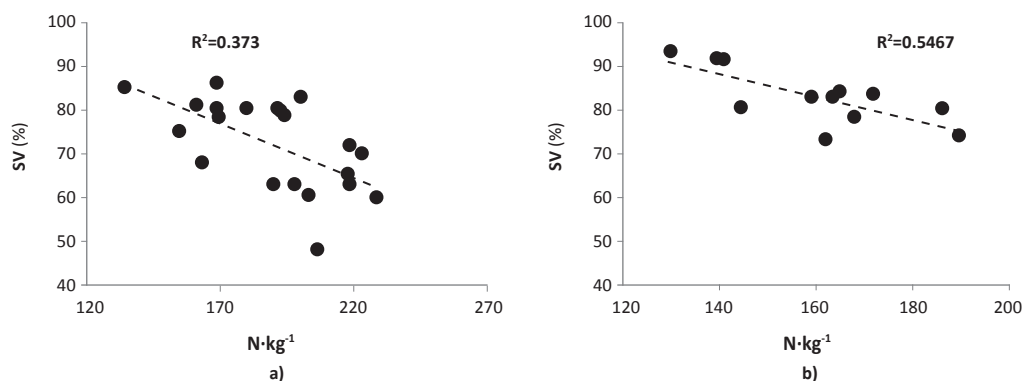
Figure 3. Correlation between mean SV and average N·kg⁻¹: **(a)** data from the FSO in Bjelovar and **(b)** data from the FSO in Garešnica.

DISCUSSION

Observations of seed yield in *Quercus robur* L. FSOs gathered during 10 years across Croatia revealed that there was no year of complete acorn absence. However, acorn yield showed considerable variations, both within and among years. Four times acorn crops were synchronized in all monitored FOs in the years 2010, 2011, 2015 and 2017 (Table 2). These results correspond to the general observations of cyclic acorn production, i.e. populations or individuals exhibited differences in a specific year, which resulted in variations in the annual acorn production (Goodrum et al. 1971, Cecich and Sullivan 1999, Healy et al. 1999, Greenberg 2000, Koenig and Knops 2002, Abrahamson and Layne 2003, Pearse et al. 2016, Bogdziewicz et al. 2018). For pedunculate oak, Gradečki-Poštenjak et al. (2011) reported significant crop year occurrence in regular intervals of every 2 to 3 years (5 mast years in a 11-year period), though quite different in quantity of crop yield. Although it is stated that the full yield follows the year in which the yield was absent or was very poor (Idžojtić 2013), our findings highlight that seed production in one year was followed sequentially by a high acorn crop in the next (in 2010 and 2011 we recorded consecutive acorn years). The results corroborate other authors who reported possible large seed crops in successive years (eg. Koenig et al. 1994). The successive high seed crops are possible also in other tree species (for example, Tapper (1992) noted an increased probability of fruiting in years following high seed production in *Fraxinus excelsior* and Norton and Kelly (1988) observed high seed production in successive years in *Dacrydium cupressinum*).

Despite the relatively large number of studies, there is limited knowledge about mechanisms and factors driving variable seed production. Allen et al. 2014 and Buechling et al. 2016 state that the initiation of masting results from a combination or sequence of climate cues. For *Q. robur* these include high spring and summer temperature within the crop years (Askeyev et al. 2005, Caignard et al. 2017, Hanley et al. 2019) and cool, wet conditions in the early autumn preceding the event (Crawley and Long 1995). In addition, masting variations within genus *Quercus* are highly site- and species-specific. For example, warm spring and summer conditions promoted increased acorn production in *Q. lobata* (Koenig et al. 2015), cool summer temperatures in *Q. macrocarpa* and warm spring temperatures 2 years prior to acorn maturation in *Q. rubra* (Koenig and Knops 2014). Summer drought, date of last spring frost and spring temperature were important for *Q. velutina* and *Q. rubra*, while for *Q. alba* spring temperature on days without rain was important (Sork et al. 1993). Consequently, there are many other factors that can influence masting besides just favourable environmental conditions. The idea of a combination of unfavourable environmental conditions (which limit reproduction), subsequent accumulation of resources (more resources spent on reproduction) and released resources for reproduction when environmental conditions are favourable is based on research conducted by Bogdziewicz et al. 2018. Also, potential factors which affect acorn crop are site and stand conditions, tree position and crown size (Dey 1995). The inherent periodicity may vary because of unfavourable temperature, precipitation in the time of flowering and the occurrence of stress caused by drought or frost periods during previous years (Harapin et al. 1996, Askeyev et al. 2005, Koenig et al. 2010). Some assumptions are the abundance of nutrients and substances required for the development of seeds, the synchronized flowering of most trees in a given locality and the lower adaptation ability of trees to pests (Sork et al. 1993, Kelly 1994). Different conclusions about the impact of factors affecting seed yield indicate the need for further research on this topic, drawing particular attention to crop quantification.

Seed quality is defined as "a measure of characteristics or attributes that will determine the performance of seeds when sown or stored" (Hampton 2002). Timing of collections can be a key factor in determining seed quality because maximum SV occur at physiological maturity (Bonner 2008). Collecting too early can results in lower seed quality due to seed immaturity, while collecting too late can also be detrimental, because seeds may be lost due to predation by animals or insects, or seed deterioration. Given the importance of acorn quality, the aim of this

research was to test the effect of seed size and MC on SV. SV is an important parameter for plant conservation and research because it denotes the degree to which the seed is alive, metabolically active and whether it possesses enzymes capable of catalysing metabolic reactions needed for germination and seedling growth. In the current study, there were no significant differences in SV among FSOs (Table 3), but SV among years within FSOs were statistically significant (Figure 1). Additional support for this conclusion is provided by studies in which considerable variability in acorn seed viability (or germination) occurs between different sites (Pritchard and Manger 1990, Phillips 1992, Gradečki-Poštenjak et al. 2018, Gradečki-Poštenjak et al. 2011, Franjević et al. 2018).

Importance of collecting larger acorns is sometimes pointed out (Matić et al. 1996c, Crnković 2004), as there is a correlation between acorn size and the height in early phases of the plants' life cycle (Kleinschmit 1993, Majer 2002, Quero et al. 2007, Landergott et al. 2012, Kesić et al. 2018). However, it is also generally accepted that larger seeds perform better than small seeds (Westoby et al. 1992). Kaliniewicz and Tylek (2018) found that the germination capacity of the smallest acorns ranged from 33% to 73%, while the largest acorns were characterized by the highest germination capacity in the range from 89% to 100%. Gradečki-Poštenjak et al. (2011) also reported a relationship between mean acorn size and mean acorn viability (yield 2006 – viability 83%, pcs·kg^{-1} 185; yield 2010 – viability 71%, pcs·kg^{-1} 203). Our research confirms these findings, i.e. lower mean number of acorns per kg correlated with higher viability (Figure 3). From this, it can be assumed that larger acorns indicate better quality, i.e. increased germination capacity. This assumption was confirmed for other tree species as well (e.g. for the *Castanea sativa* (Cicek and Tilki 2007)). Contrary, for some tree species germination characteristics were not significantly influenced by seed size and weight (e.g. for mountain hemlock (Edwards and El-Kassaby 1996) or *Quercus libani* (Alptekin and Tilki 2002)).

Acorns of the highest quality have MC of 40–45% fresh weight (Suszka et al. 2000, Szabla and Pabian 2009). Doody and O'Reilly (2008) reported that freshly harvested acorns had 46–48% of MC (about 24 h after harvesting). Özbingöl and O'reilly (2005) reported that acorn mean MC at delivery time was 42–43%, while Chmielarz et al. (2022) state that the mean MC of the acorns upon delivery was 37.7% and 40.8% of fresh weight for different seed lot. In our study, the mean MC of the acorns at delivery time was 39.6%, ranging from 33.1% to 44.1% (Table 2). These results were slightly lower than the previously reported research. The reasons for this were probably the storage conditions and time lag between the collection and transfer to the laboratory. MC of acorns at the time of shedding varies from 30% to 55% depending on the species (Bonner 1974b, Gosling 1989, Sobrino-Vesperinas and Viviani 2000, Joët et al. 2013), but it

rapidly falls, which is primarily due to desiccation (Pritchard 1991, Gosling 1989, Connor and Sowa 2003). For example, in *Q. coccifera* and *Q. pubescens* viability was lost when MC dropped below 26% (Ganatsas and Tsakaldimi 2013), 15% in *Q. nigra* (Bonner 1996), and 22% in *Q. alba* (Connor et al. 1996). Earlier studies showed that the critical MC for pedunculate oak seeds was approximately 40%, below which viability declined rapidly (Suszka and Tylkowski 1980, Gosling 1989, Poulsen and Eriksen 1992). Suszka (2002) found that acorns dried to a MC below 22% did not germinate. Our results also clearly showed decreasing acorn SV due to lower MC (Figure 2). On average, viability drops below 70% when acorn mean moisture decrease below 36%.

CONCLUSIONS

The results of this study provide information on the seed yield from *Quercus robur* seed objects across Croatia in 10 years and the effects of seed size and seed moisture content on seed viability. Acorn production was very variable, with a considerable inter-annual variability in seed production and variability among seed objects within the same year. In the study period, four years were identified as years with higher number of samples. The results of testing seed viability showed significant variations among years within a forest seed object, but no difference in seed viability among seed objects. There were significant positive correlations between the impact of acorn size as well moisture content on seed viability.

Conflicts of Interest

The authors declare no conflict of interest.

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A Parsimonious Generalised Height-Diameter Model for Scots Pine Plantations in Bulgaria: a Pragmatic Approach

Tatiana Vassileva Stankova[1,*], Angel Ferezliev[2], Dimitar N. Dimitrov[1], Proletka Dimitrova[1], Penka Stefanova[1]

(1) Forest Research Institute, Bulgarian Academy of Sciences, 132 "St. Kliment Ohridski" blvd., 1756 Sofia, Bulgaria; (2) Experimental Station on Coniferous Forests - Velingrad, Forest Research Institute, Bulgarian Academy of Sciences, 44 Khan Asparuh Blvd., 4600 Velingrad, Bulgaria

* Correspondence: e-mail: tatianastankova@yahoo.com

ABSTRACT

Considering the state-of-the-art of forest inventory in Bulgaria, our investigation pursued development of a parsimonious generalised height-diameter model for the Scots pine plantations in the country. A number of 2-, 3- and 4-predictor candidate models were examined and compared based on their goodness-of-fit statistics. Data records obtained in variable-sized sample plots, established throughout the distribution range of the plantations and covering the variety of sites, densities and growth stages were used to fit the models. Two hundred twenty-four plot-level measurements and 3056 tree height-diameter pairs were utilised for parameterization. An independent data set of tree-level measurements and two sets of dominant height-diameter pairs, estimated for differently defined top height tree collectives, were used for model validation. Statistical analyses were carried out using packages nlstools, moments, equivalence, car, nlme, stats and the results were illustrated with ggplot2 and graphics packages of R software environment. A modified form of Gaffrey's model was selected, which estimates the height of a tree through the breast-height tree diameter, mean stand height and diameter, and accounts for the tree social status. It was fitted by generalised non-linear least squares method, with residual variance weighted by a product of tree diameter and mean stand height exponential functions. An adjusted coefficient of determination of 0.917 and residual standard error of 0.794 m indicated the high predictive potential of the derived model. Validation tests showed that the estimated regression line is very well fitted to the independent data and is appropriate to forecast dominant stand heights. The range of errors, relative to the predicted dominant height values, was narrow, ±25-30%, with low magnitude of the average of their absolute values (4-5%). The equivalence tests rejected the null hypothesis of dissimilarity regarding model bias (observations-predictions line intercept) for all validation data sets, for a region of equivalence as narrow as ±5%. The 3-predictor generalised height-diameter model developed in our study needs information readily available from the inventories and therefore can be broadly used. Its application in dominant stand height prediction is recommended.

Keywords: *Pinus sylvestris* L.; Gaffrey's function; generalised non-linear least squares; equivalence test; dominant stand height; mean stand height

INTRODUCTION

Total height is an important variable of the trees that is employed in estimation of stand volume and site quality and is used in the description of the vertical stand structure. Measuring tree heights costs time and effort and the foresters usually welcome an opportunity to calculate this variable with an acceptable accuracy (Temesgen and Gadow 2004). Simple linear or non-linear relationship to tree diameter usually fulfils the requirement for adequate tree height estimation at stand level ("local height-diameter model"), but expanding the predictions to a wider region would not be so trustworthy, given the dependence of the model on the growth conditions and stand characteristics. Indeed, the

trees growing at higher densities will probably have smaller diameters than those in less dense stands due to competition (i.e. density-dependence), trees having the same diameter at different times will belong to sociologically different classes (i.e. age-dependence) and the height curves for good quality sites are expected to have steeper slopes than those for poor quality sites (i.e. site quality-dependence) (López Sánchez et al. 2003). Analysis of the ecoregion-based height-diameter models for white spruce in Alberta's boreal forests has suggested that the height-diameter relationships are different in different ecoregions, probably because of the very different biogeoclimatic conditions (Huang et al. 2000). The authors found that applying a height-diameter model fitted from one ecoregion to different ecoregions resulted in overestimations between 1.10% and 29.05%, or underestimations between 1.92% and 21.92%. Generalized model forms and mixed-effects modelling are usually applied to localize the height-diameter relationship to specific stands (Weiskittel et al. 2011). Referring to earlier investigations, Crecente-Campo et al. (2010) generalised that the incorporation of stand variables in a local height-diameter model reduces bias and increases precision. Ahmadi and Alavi (2016) concluded that the inclusion of stand characteristics improved the prediction accuracy of tree height estimation for *Fagus orientalis* Lipsky trees, while Staudhammer and LeMay (2000) found that the introduction of stand density variables resulted in increased accuracy for predicting heights of alder. Temesgen and Gadow (2004) reported that the expansion of the simple height-diameter relationships with stand-level attributes reduced the root mean square errors (RMSE) by 30.0 cm. Temesgen et al. (2007), who developed regional height-diameter equations for major tree species of southwest Oregon, estimated decrease in the RMSE of the expanded models by a minimum of 6.1% to a maximum of 22%.

By definition, the mean stand height corresponds to the quadratic mean diameter (or mean basal area diameter), due to its practical importance in volume calculation, and therefore its estimation is straightforward. The definition and, consequently, the estimation of the dominant stand height, however, may vary widely. While Pretzsch (2009) limits the definition for dominant height to 3 versions, according to the top height tree collective defined, Van Laar and Akça (2007) mention 6 different measures of stand dominant height. Poryazov (2009) registered 19 different ways to define and calculate dominant stand height. In Bulgaria, similar to other countries such as Estonia (Tarmu et al. 2020), mean stand height is the measure commonly used in the forest management practice and there is no standard or officially accepted protocol for dominant height estimation. Duhovnikov (1972) determined dominant stand height as the height corresponding to the average diameter of the 20% thickest trees in the stand, while Shikov (1974) and Ferezliev and Tsakov (2010) calculated dominant height as an arithmetic average of the heights of the 100 thickest trees per hectare. Petrin (1987) and Tonchev (2022), on the other hand, applied the definition of dominant height as the one corresponding to the quadratic mean diameter of the 100 thickest trees per hectare. Stankova et al. (2006) derived a relationship of the dominant on mean stand height of Scots pine plantations, using sub-samples of total height measurements, taken on 20% of the trees in the plots that represent the average and dominant diameter trees in equal proportions (i.e. dominant height corresponding to

the average height of the 10% thickest trees in the plot). It becomes clear that, for situations where a standard procedure for dominant height estimation is not established, such as in Bulgaria, a height-diameter relationship based on dominant stand height as a predictor would be of limited applicability. A generalised height-diameter model, based on tree diameter, dominant stand diameter and height, stand age and density has been developed for Scots pine plantations (Stankova and Diéguez-Aranda 2013). Its confident application, however, assumes sufficient precision of the stand age estimate and requires additional field-collected data on stand density, as advised by Stankova (2012). In addition, the aforementioned lack of coherence in dominant height estimation in Bulgaria casts doubt on both the consistency of the data used for parameterization and the reliability of the model predictions for independent data sets.

The national forest inventories in Bulgaria are characterised as stand-wise inventories for local forest management planning (FMP) (Groen et al. 2013), which are implemented by management units at approximately 10-year intervals. The inventory description of each forest stand includes several principal quantitative parameters: stand age (years), basal area (m²·ha⁻¹), standing stock (m³·ha⁻¹), quadratic mean diameter (cm), mean stand height (m), stocking rate, relative to "normal" (fully stocked) stand (0-1) and site index expressed as a categorical variable in the range I-V and based on functional relationship of mean height to age. Stand density, expressed by the number of trees per hectare, is rarely recorded and the highest precision for stand age is usually 5 years. According to the Ordinance for inventory and planning of the forest territories of Bulgaria, in the vast majority of cases (stand types by age classes) estimates of the stand basal area and volume are obtained from the growth and yield tables according to the in situ evaluations of mean height, age and stocking rate. The specifics of forest inventory in Bulgaria suggest that the stand-level parameters that are of sufficient precision and are readily available for implementation into generalized height-diameter functions are the average stand height and diameter, and perhaps basal area.

In forest inventories, the mean stand height is needed to estimate the stand volume, while dominant stand height is considered a more suitable measure for predicting site quality, because it is less sensitive to thinnings (Van Laar and Akça 2007, Tarmu et al. 2020). Consequently, the accuracy of the dominant stand height predictions by a height-diameter model is important. Sharma et al. (2002), who investigated top height as defined in 7 different ways, found out that for both thinned and unthinned loblolly pine plantations, there were significant differences in the respective top height estimates. Moreover, with the exception of a few cases at certain measurements, the site index predictions based on them also differed significantly. Therefore, it is important to assess also the height-diameter model accuracy in predicting stand dominant height defined in various ways, i.e. from differently assembled largest trees collectives.

The main objective of our study was to derive a practically-oriented, parsimonious generalised height-diameter model for Scots pine plantations in Bulgaria and to examine and validate its applicability for prediction of tree height, particularly of the largest trees that form the collective for the estimation of dominant stand height.

MATERIALS AND METHODS

Data Sets

The data set used to derive the height-diameter relationship of the trees in the Scots pine plantations in Bulgaria was generated from both personally collected and published data records. These records were obtained in variable-sized sample plots (Table 1) of circular or rectangular form, which were established randomly throughout the distribution range of the plantations, with a primary criterion to cover the variety of sites, densities and growth stages of the Scots pine plantations in Bulgaria. One hundred and twenty-one of the plot-level measurements were collected in once-measured plots, 111 of which were personally established and 10 were inventoried by other researchers (Efremov 2006). The remaining 103 plot-level data records were provided from the Appendices to the Forest Inventory Plans and other published data sources (Marinov 2008) and comprised data from permanent sample plots measured 1 to 4 times. Beside the total height (h, m) - breast-height diameter (d, cm) measurements of trees, sampled in random or systematic manner in each plot, the stand-level parameters: basal area (G, $m^2 \cdot ha^{-1}$), stand density (N, $trees \cdot ha^{-1}$), quadratic mean diameter (D_m, cm) and mean stand height (H_m, m) were used to fit the regression models.

Table 1. Description of the data used for parameterization and validation of the height-diameter relationship of Scots pine plantations in Bulgaria.

Data Set	Variable		Mean	Standard deviation	Minimum	Maximum
Parameterization Data Set PM=224, *n*=3056	Stand level	D_m (cm)	18.1	6.5	3.6	35.3
		H_m (m)	16.3	4.7	3.2	29.0
		N ($trees \cdot ha^{-1}$)	2236	1498	421	8210
		G ($m^2 \cdot ha^{-1}$)	42.76	11.80	6.10	72.25
		PS (m^2)	620	502	85	1989
	Tree level	h (m)	16.4	4.5	2.9	35.0
		d (cm)	18.7	7.1	4.0	47.0
Validation Data Set 1 (Valid.Data) PM=46, *n*=329	Stand level	D_m (cm)	13.6	6.6	4.0	26.0
		H_m (m)	12.2	5.4	3.3	23.2
		N ($trees \cdot ha^{-1}$)	3793	2501	1042	12200
		G ($m^2 \cdot ha^{-1}$)	39.30	16.69	5.55	71.03
		PS (m^2)	232	160	45	576
	Tree level	h (m)	13.7	5.1	2.9	23.8
		d (cm)	18.9	8.8	4.0	42.0
Validation Data Set 2 (to compile Dom.10perc) PM=111, *n*=1387	Stand level	D_d (cm)	22.7	7.9	6.7	43.1
		H_d (m)	17.1	5.4	3.6	32.6
		D_m (cm)	16.9	6.8	2.5	35.3
		H_m (m)	15.9	5.2	3.2	32.0
		N ($trees \cdot ha^{-1}$)	2652	1663	613	8210
		G ($m^2 \cdot ha^{-1}$)	44.56	12.32	6.10	72.25
		PS (m^2)	299	211	85	1042
	Tree level	h (m)	16.9	5.0	2.9	35.0
		d (cm)	20.0	7.5	4.0	47.0
Validation Data Set 3 (to compile Dom.D0H0) PM=100, *n*=1292	Stand level	D_0 (cm)	22.3	6.4	7.0	42.3
		H_0 (m)	16.6	4.7	4.0	27.2
		D_m (cm)	15.7	5.8	2.5	32.8
		H_m (m)	15.1	4.5	3.2	26.5
		N ($trees \cdot ha^{-1}$)	2854	1628	825	8210
		G ($m^2 \cdot ha^{-1}$)	44.29	12.45	6.10	72.25
		PS (m^2)	265	191	85	1042
	Tree level	h (m)	16.4	4.6	2.9	30.0
		d (cm)	19.1	6.7	4.0	47.0

Abbreviations: H_m - mean stand height (m), D_m - quadratic mean diameter (cm), G - stand basal area ($m^2 \cdot ha^{-1}$), N - stand density ($trees \cdot ha^{-1}$), D_d - top stand diameter (cm) estimated as the arithmetic average of the breast-height diameters of the 10% thickest trees in the plots of Validation Data Set 2, D_0 (cm) dominant stand diameter (cm) estimated as the quadratic mean of the breast-height diameters of the 100 thickest trees per hectare (recalculated according to the plot size) in the plots of Validation Data Set 3, H_d - top stand height (m) estimated as the arithmetic average of the heights of the 10% thickest trees in the plots of Validation Data Set 2, H_0 - dominant stand height (m) estimated and as the Lorey's mean of the heights of the 100 thickest trees per hectare (recalculated according to the plot size) in the plots of Validation Data Set 3, h - tree height (m), d - diameter at breast height (cm), PM - total number of combinations of plot-measurement occasions, i.e. plot-level measurements, n - number of trees measured for heights, PS - plot size (m^2).

In addition to the parameterization data set, we employed 3 data sets for model validation (Table 1). The first of them, Validation Data Set 1, was composed of completely independent data, personally collected in 46 plots measured once, following the same principle of plot distribution as of the parameterization data set. The sub-samples of fewer tree heights measured per plot (Table 1) were not appropriate to estimate dominant heights for differently defined largest tree collectives and therefore the applicability of the model was only examined with the 329 pairs of tree-level height-diameter measurements recorded (abbreviated as "Valid.Data" in the results). The second and the third sets for validation were created from subsets of the parameterization data set, which allowed dominant height estimation from differently assembled largest tree collectives. Two types of largest tree groups were compiled. The tree-level data of Validation Data Set 2 was used to calculate the arithmetic averages of the tree diameters and heights of the 10% thickest trees in the plot (as estimated in Stankova et al. 2006), referred hereafter as "top diameter" and "top height" and abbreviated as D_d and H_d. The tree-level data of Validation Data Set 3 were used to estimate the quadratic mean diameter of the 100 thickest trees per hectare, recalculated according to the plot size, and its respective basal area-weighted (Lorey's) height, referred hereafter as "dominant diameter" and "dominant height" and abbreviated as D_0 and H_0. The ability of the height-diameter relationship to predict the top/dominant stand height from the top/dominant stand diameter was tested with 111 top height-top diameter pairs (abbreviated as "Dom.10perc" in the results) and 100 dominant height-dominant diameter pairs (abbreviated as "Dom.D0H0" in the results), which were able to be extracted from, respectively, Validation Data Set 2 and Validation Data Set 3 (Table 1).

Model Development, Estimation and Validation

Considering the state-of-the-art of forest inventory in Bulgaria, we concluded that the stand-level parameters which are the most appropriate to be used as predictors in the generalized height-diameter functions are the average stand height and diameter, because they are measured during the inventories and are available in the inventory descriptions of the stands. Generalised height-diameter models that include mean stand diameter and height (or dominant stand diameter and height) are usually derived from simple height-diameter relationships by formulating a second equation of the same functional form, but constrained to pass through the mean (or dominant) stand height and mean (or dominant) stand diameter. The system of the two equations is then solved for tree height, converting the simple one-predictor relationship into a 3-predictor regression model (Mønness 1982, Krumland and Wensel 1988, Cimini and Salvati 2011). Alternatively, a generalized height-diameter model can be derived by expressing the parameters of a simple relationship as functional forms of the selected stand-level variables (Harrison et al. 1986, Castedo-Dorado et al. 2001). A number of such 3-predictor height-diameter models, which are based on tree diameter, mean (or dominant) stand height and mean (or dominant) stand diameter and their modifications, developed from simple predecessor functions that have shown appropriate for modelling the height-diameter relationship, can be

found in the literature (e.g., López Sánchez et al. 2003, Lei et al. 2009). We classified the functions that we extracted into 3 groups: 1) based on D_m and H_m (Table A1); 2) based on D_0 and H_0 (Table A2) and 3) based on D_m and H_0 (Table A3). Two of the third-group relationships, by Harrison et al. (1986) and by Gadow and Hui (1999), did not include a stand-level diameter measure as an independent variable, i.e. they described 2-predictor functions. We examined the models from the first group (7 models) in their original form. The second group formulations (18 models) were tested keeping their functional forms, but replacing D_0 and H_0 by D_m and H_m, considering that the second group models were derived in an analogous manner to the first group relationships. We fitted the relationships from the third group (7 models) in their base form after substituting H_m for H_0, but we also examined their expanded forms where H_0 was substituted by a function of H_m. We assumed 2 different forms of the relationship: linear function of H_m ($H_0=a_0H_m+a_1$), as it has been often approximated for practical application (M.L.W.F. 1980, Mihov 1986, López Sánchez et al. 2003) and non-linear function of H_m and N ($\ln(H_0/H_m)=a_0[\ln(N/150)]^{a_1}$), as suggested by García (2017). Given the importance of basal area as a measure of stand stocking, we considered the possibility for its incorporation into the generalised height-diameter model as well. Therefore, we examined eight more relationships (Table A4) that included in their originally published form tree diameter, measures of stand stocking (basal area and/or stand density) and, in some cases, stand-level measures of height and diameter (mean or dominant).

We first fitted the formulated generalised height-diameter relationships by non-linear least squares (NLS) method, we checked if the assumptions to the residuals were met and we examined the goodness-of-fit of the regression models. Homoscedasticity of errors was evaluated by exploring the plots of residuals against the independent variable values and against the predicted values. In case of diagnosed heteroscedasticity of errors, that is often the case for biological data, the model was not rejected, but the option to resolve the issue through refitting by generalised non-linear least squares (GNLS) method was assumed. The data from the Scots pine plantations in Bulgaria have generally revealed a tendency to leptokurtic residual distributions for most of the elaborated biometric models (e.g., Stankova and Diéguez-Aranda 2013, 2017, 2020). The relative robustness of the regression analyses against even considerable departures from normality has been considered, provided that the sample size is sufficiently large (Frost, 2014) or the residual distribution is not severely asymmetric (Sokal and Rohlf 1995). Therefore, we examined the normality of errors by separate tests for skewness and kurtosis and we discarded those tested models that showed significantly skewed residual distributions as assessed by D'Agostino's skewness test (D'Agostino 1970). In addition, we visually inspected the histograms of the residual distributions as well as the quantile-quantile plots.

Thereafter, the models were tested for bias by a *t*-test for mean error equal to zero and simultaneous *F*-test for slope equal to 1 and zero intercept of the linear regression relating the observed and predicted values. Stability of parameter estimates was assessed by the Percent Relative Standard Error (PRSE%), which is the ratio (in percent) between the standard error and the absolute value of the

regression parameter and must attain values below 25%. The PRSE% served indirectly also to control the effect of possible outliers and influential observations (Sileshi 2014). Collinearity was handled through the condition number that must obtain estimates below 30.

All models that proved adequate were compared by their regression statistics adjusted coefficient of determination (Adj. R^2), residual standard error (RMSE, m) and Akaike Information Criterion (AIC). To select the relationship best suited to the data of our study, we employed Akaike weights (AIC weights, %) that represent the relative likelihoods of the models, providing strength of evidence in favour of one model over the other (Wagenmakers and Farrell 2004, Sileshi 2014). In case of diagnosed heteroscedasticity of errors, the selected model was refitted by the generalised nonlinear least squares method. Variance-correcting functions, based on combinations of different functional forms of the predictors, were tested and compared in consideration of both residual homoscedasticity and overall goodness-of-fit of the regression model. The improvement of the residual variance and the model adequacy by GNLS as compared to NLS was assessed by the residual plots, and the model predictive abilities, as fitted by the alternative methods (NLS vs. GNLS), were contrasted by a likelihood ratio test.

To validate the newly developed model, we fitted it to each validation data set separately and estimated several test statistics (formulae shown under Table 4). Model efficiency (ME), which is a relative measure of model performance similar to the coefficient of determination, was assessed. The quartiles of the relative error distributions as well as the averages of the absolutes values of these errors were evaluated, which indicate the range and the magnitude of prediction errors relative to the predicted heights. Finally, to compare the observed with the predicted values, we performed equivalence tests (Robinson et al. 2005, Weiskittel et al. 2011) which allow goodness-of-fit judgment by combining test of model bias, assessing equality of means (test for the intercept) and test of model proportionality, evaluating similarity of individual observations (test for the slope). The regions of equivalence were set as narrow as ±5% to be compared with the approximate joint two one-sided 95% confidence intervals for the slope and intercept for 100 bootstrap replicates.

Statistical analyses were carried out using packages nlstools, moments, equivalence, car, nlme, stats and the results were illustrated with ggplot2 and graphics packages of R software environment (Baty et al. 2015, Komsta and Novomestky 2015, Robinson 2016, Wickham 2016, Fox and Weisberg 2019, Pinheiro et al. 2021, R Core Team 2021).

RESULTS

One model with two independent variables, eight three-predictor models and two models with four independent variables met the goodness-of-fit criteria (Table 2). Nine of these 11 models came from the third group of tested relationships that originally included dominant height and quadratic mean diameter as stand-level predictor variables (Table A3). The model by Harrison et al. (1986) (abbreviated "Harr" in Table 2) was the only 2-predictor model that

fitted our Scots pine data and did not include a stand-level diameter measure. It was parameterized successfully after substitution of dominant stand height by mean height, while its expanded model forms failed to converge. The model by Gaffrey (1988) as well as its modification by Diéguez-Aranda et al. (2006) (Gafr2 and Gafr1 in Table 2, respectively) were fitted successfully when the dominant stand height was substituted by a linear function of mean height. Two expanded forms of the model by Pienaar et al. (1990) also showed adequate. The first of them was a function with three independent variables, where dominant height was presented by a linear relationship of the mean height (Pien1 in Table 2) and the second function had four predictors, where a product of mean height with density-based term was considered instead: $H_m e^{[\ln(N/150)]a_1}$ (Pien2 in Table 2). The model by Shröder-Álvarez-González (2001) and its predecessor, the model by Mirkovich (1958), supplied more adequate regressions: derived by a simple substitution of dominant with mean stand height (Mir1, ShrA1 in Table 2) or by incorporation of a product of mean stand height with power term of density – $H_m(N/150)^{a_0}$ – on its place (Mir2, ShrA2 in Table 2). Finally, a model by Sharma and Parton (2007) including the ratio N/G (Table A4, ShaPar2 in Table 2) as well as its modified form where this ratio was replaced by the quadratic mean diameter (Table A2, ShaPar1 in Table 2) were also elected as possible candidates to describe the height-diameter relationship of the trees in the Scots pine plantations in Bulgaria. All adequate generalized models showed high coefficients of determination (above 0.9 in most of the cases) and root mean squared errors in a narrow range: 1.29 – 1.47m (Table 2). However, the model Gafr2, which was based on the function by Gaffrey (1988), proved superior to the other compared models, as indicated by Akaike weights (Table 2).

Unbiased, symmetric residual distribution was revealed by both graphical and analytical tests for this model, but heteroscedasticity of residuals was diagnosed (Figure 1). To assure higher precision of the parameter estimates as well as their standard errors, generalized non-linear least squares method was applied. A variance function that was the product of exponential relationships to tree diameter and mean stand height was selected and applied (Table 3). The likelihood ratio test indicated an increased predictive power of the model fitted via generalized non-linear least squares (Table 3) and the plots showed an improvement in the residual variance and model adequacy (Figures 1 and 2). Parameter Relative Standard Errors (PRSE%) attained values below 20%, assuring the stability of the parameter estimates (Table 3).

High rates of model efficiency were calculated for all examined validation data sets (Table 4). They showed that the estimated regression line is very well fitted to the independent data and is appropriate to forecast top and dominant stand heights. The range of errors, relative to the predicted dominant and top height values, was narrow, ± 25 – 30% (Table 4), with low magnitude of the average of their absolute values (4 – 5%). A tendency to underestimate the tree heights at the upper size range and overestimate those at the lower size range for the independent validation data can be seen in Table 4 and Figure 3A. It was asserted also by the equivalence test, which showed that the hypothesis for
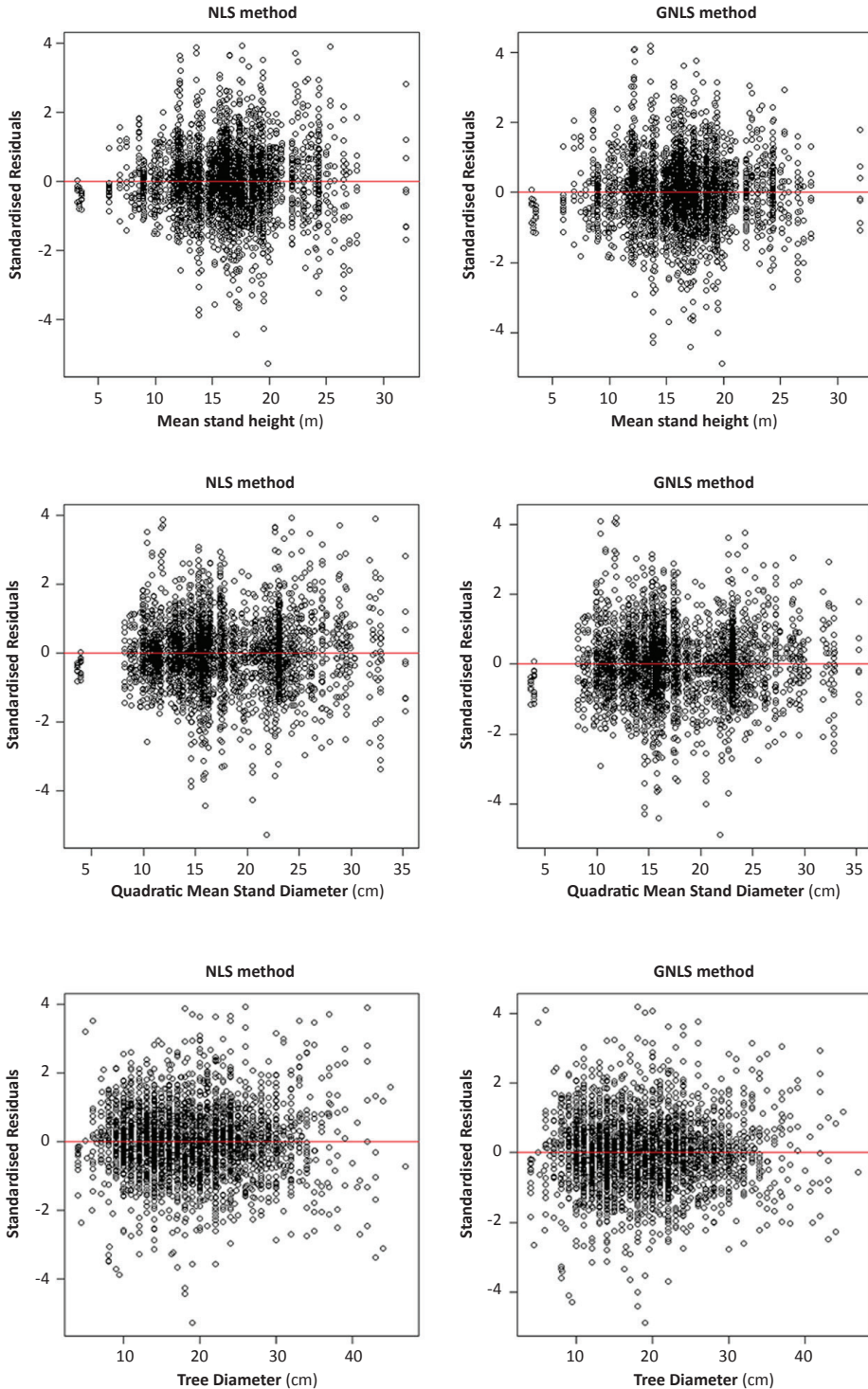
Figure 1. Plots of standardised residuals against the independent variables for the selected model Gafr2, fitted by non-linear least squares method (NLS method) and generalised non-linear least squares method (GNLS method).

Table 2. Comparison of the adequate height-diameter models of trees in Scots pine plantations in Bulgaria.

	Model	RMSE (m)	Adj. R²	AIC	AIC weights (%)
Harr	$h = H_m(1 + b_0 e^{b_1 H_m}) \cdot (1 - e^{-b_2 d/H_m})$	1.474	0.891	11047.71	0.00
Gafr1	$h = 1.3 + (a_0 H_m + a_1) \cdot e^{b_0(1/D_m - 1/d)}$	1.320	0.913	10375.97	0.00
Gafr2	$h = 1.3 + (a_0 H_m + a_1) \cdot e^{b_0(1-D_m/d) + (1/D_m - 1/d)}$	1.285	0.917	10209.94	99.89
Mir1	$h = 1.3 + (b_0 + b_1 H_m - b_2 D_m) \cdot e^{-b_3/d}$	1.346	0.909	10495.77	0.00
Mir2	$h = 1.3 + (b_0 + b_1 H_m(N/150)^{a_0} - b_2 D_m) \cdot e^{-b_3/d}$	1.343	0.910	10482.02	0.00
Pien1	$h = (a_0 H_m + a_1) \cdot (1 - e^{-b_1 d/D_m})^{b_2}$	1.288	0.917	10223.59	0.11
Pien2	$h = b_0 H_m e^{[\ln(N/150)]^{a_1}} \cdot (1 - e^{-b_1 d/D_m})^{b_2}$	1.291	0.917	10239.47	0.00
ShrA1	$h = 1.3 + (b_0 + b_1 H_m - b_2 D_m) \cdot e^{-b_3/\sqrt{d}}$	1.322	0.912	10383.18	0.00
ShrA2	$h = 1.3 + (b_0 + b_1 H_m(N/150)^{a_0} - b_2 D_m) \cdot e^{-b_3/\sqrt{d}}$	1.317	0.913	10361.91	0.00
ShaPar1	$h = 1.3 + b_0 H_m^{b_1} \cdot (1 - e^{-b_2 d \cdot D_m^{b_3}})^{b_4}$	1.290	0.917	10234.47	0.00
ShaPar2	$h = 1.3 + b_0 H_m^{b_1} \cdot (1 - e^{-b_2 d(N/G)^{b_3}})$	1.290	0.917	10234.16	0.00

Abbreviations: h - tree height (m), d - diameter at breast height (cm), H_m - mean stand height (m), D_m - quadratic mean diameter (cm), G - stand basal area (m²·ha⁻¹), N - stand density (trees·ha⁻¹), a_0, a_1, b_0, b_1, b_2, b_3, b_4 - model parameters; RMSE - Residual standard error, m; Adj. R² - adjusted coefficient of determination; AIC - Akaike Information Criterion; AIC weights - Akaike weights

Table 3. Regression estimates and goodness-of-fit statistics of the selected height-diameter model, fitted by generalized non-linear squared method.

Likelihood Ratio Test						
Method	AIC	BIC	Degrees of freedom	logLik	Likelihood Ratio	p-value
GNLS	10131.67	10167.82	6	-5059.837		
NLS	10209.94	10234.04	4	-5100.971	82.2677	<0.001
Regression estimates and goodness-of-fit test statistics						
Adj. R²	Variance function			Regression Parameters		
0.917	exp(2θd)exp(2ηH_m)			a_0	a_1	b_0
	Variance Function Parameters		Estimate	0.968	-0.748	0.320
RMSE	θ	η	SE	0.005	0.083	0.007
0.794	0.003	0.026	PRSE %	0.543	11.122	2.091

Abbreviations: GNLS - generalized non-linear least squares, NLS - non-linear least squares, AIC - Akaike Information Criterion, BIC – Bayesian Information Criterion, logLik – log-likelihood, Adj. R² - adjusted coefficient of determination, RMSE - Residual standard error (m), d - diameter at breast height (cm), H_m - mean stand height (m), a_0, a_1, b_0, θ, η - model parameters, SE - standard error, PRSE % - Parameter Relative Standard Error (%).
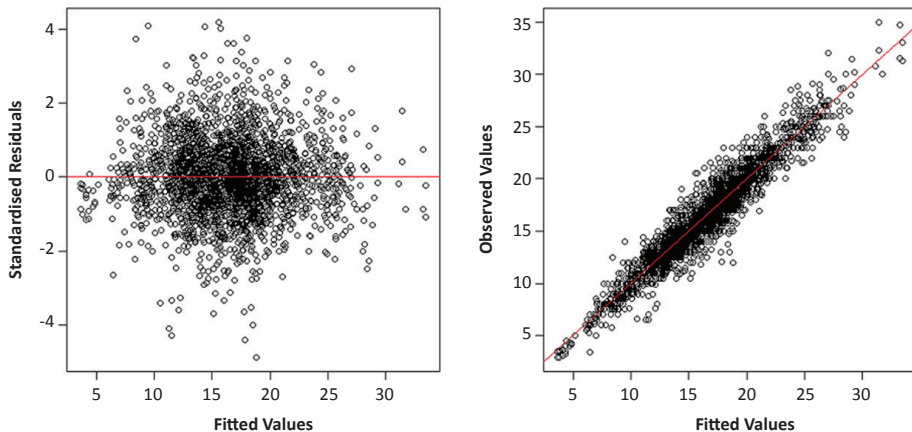

Figure 2. Plot of standardised residuals against fitted values and plot of observed values against fitted values for the selected model Gafr2, fitted by generalised non-linear least squares method (GNLS method).

dissimilarity of the slope was not rejected for this data set for ±5% region of similarity. However, as seen in Figure 3A, the lower-size range of this validation data set is underrepresented in the data set used for model parameterization. In addition, the range of the stocking rates of the sampled plantations in Validation Data Set 1 exceeds that of the parameterization data (see minimum basal area and maximum stand density in Table 1); both disparities could have caused the validation outcome. Further investigation showed that, given the ±5%

region of similarity for the intercept, the smallest region of indifference that would reject the null hypothesis of dissimilarity of the slope is ±12%, which suggests acceptable accuracy. The residual errors of the predicted dominant and top stand heights, on the other hand, are symmetrically distributed across the range of the predicted heights (Figures 3B, 3C) and equivalence tests confirmed rejection of the hypotheses of dissimilarity for both model bias and model proportionality (Table 4).
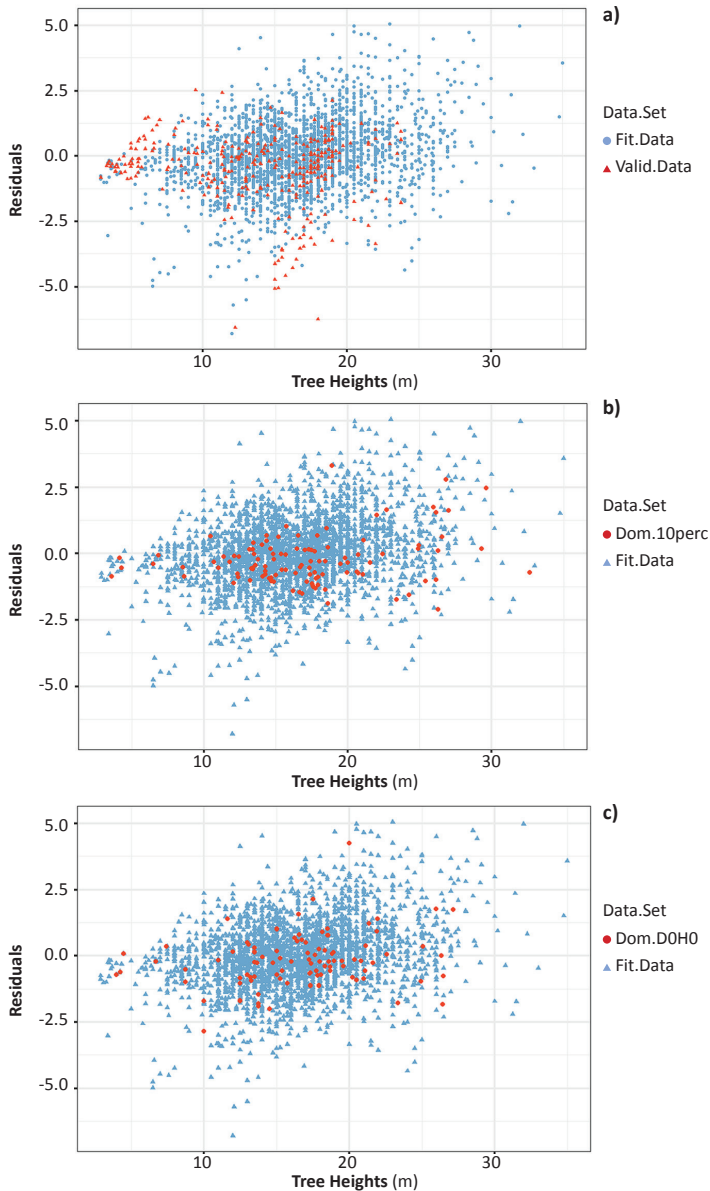

Figure 3. Plots of residuals against the observed values for parameterization and validation data sets.

Table 4. Validation statistics.

Validation data set	ME	MARE %	Perc0	Perc25	Perc50	Perc75	Perc100	Equivalence Test*	
								Slope	Intercept
Valid.Data	0.922	7.46	-53.68	-8.56	-2.33	2.25	26.56	0.89 - 0.94 (0.95 - 1.05)	13.58 - 13.85 (13.48 - 14.90)
Dom.10perc	0.968	4.50	-23.77	-5.92	-2.98	0.75	17.58	0.98 – 1.04 (0.95 – 1.05)	16.95 - 17.23 (16.50 - 18.24)
Dom.D0H0	0.953	5.11	-28.54	-5.55	-1.29	1.68	21.31	0.97 – 1.05 (0.95 – 1.05)	16.41 – 16.81 (15.94 – 17.62)

Abbreviations: Valid.Data – 329 pairs of measurements of tree height and diameter, Dom.10perc – 111 pairs of estimations of plot top height and diameter, calculated as arithmetic averages from the 10% thickest trees, Dom.D0H0 – 100 pairs of estimations of plot dominant height and diameter, calculated from the 100 thickest trees per hectare (recalculated according to the plot size) as quadratic mean diameter and basal area -weighted (Lorey's) height, ME - model efficiency, $ME=1-\frac{\sum(y_i-\hat{y}_i)^2}{\sum(y_i-\bar{y})^2}$, ARE% - absolute value of the relative error, $ARE\%=\frac{|y_i-\hat{y}_i|}{y_i}100$, where y_i, $\hat{y}_i$ are experimental and predicted height values of the i-th measurement and $\bar{y}$ represents the mean observed height value; MARE% is the average of the absolute values of the relative errors; Perc0, Perc25, Perc50, Perc75, Perc100 denote the 0th, 25th, 50th, 75th and 100th percentile of the relative errors $RE\%=\frac{y_i-\hat{y}_i}{y_i}100$, respectively.

* The approximate joint two one-sided 95% confidence intervals for the slope and intercept are shown and the ±5% intervals of equivalence beneath them in parentheses.

DISCUSSION

Eleven 2-, 3- and 4-predictor models proved adequate to fit the examined height-diameter relationship, with coefficients of determination and residual standard errors varying within narrow ranges. Comparisons of various equations, regarding height-diameter modelling, across multiple species have shown that most of them predict with a similar degree of precision when extensive data are available (Huang et al. 1992, Sonmez 2009, Weiskittel et al. 2011). Stand density (trees·ha⁻¹) is considered the most obvious factor affecting the height-diameter relationship (Crecente-Campo et al. 2010) and stand basal area (m²·ha⁻¹) is regarded as another measure of stand stocking (Clutter et al. 1983). A study by Nguyen et al. (2019) indicated that the height-diameter relationship of *Pinus koraiensis* Sieb. et Zucc. differed between stand density levels and therefore could be conditioned on stand density. An option to estimate stand density from the distance to the 3rd neighbour (Priesol 1970, Anuchin 1977) is suggested by the regulations in Bulgaria for the inventory of relatively homogenous even-aged stands, such as the Scots pine plantations. Both stand stocking parameters were considered as predictors in the generalised model formulations that we tested and were included in four of the selected relationships. Studies by other investigators proposed modelling of dominant stand height as a function of mean stand height and density (García 2017, Tarmu et al. 2020), although other results revealed that the mean-dominant height relationship can often be handled only through the functional form of a simple model (e.g., M.L.W.F. 1980, Van Laar and Akça 2007). Staudhammer and LeMay (2000), who developed a generalised height-diameter model of improved accuracy for alder after inclusion of density as an independent variable, explained the observed result with the shade-intolerance of the species coupled with rapid early growth and restricted longevity.

The final comparison of the 11 candidate models of our study elected as the most adequate a modification of Gaffrey's model (Gaffrey 1988) that included two stand attributes: mean stand height and diameter. Adamec (2015) pointed out the advantageous application of such model as compared to the elaboration of a local height-diameter relationship: the number of measured heights necessary to determine the mean height is lower than the total number of measured heights needed to fit the stand-level height curve. Mean stand height can also be regarded as an indicator of the growth stage of an even-aged stand. Unlike age, larger mean height reflects not only the time since the plantation establishment, but also better site quality on the one hand and stronger growth potential due to genetic factors (intrinsic growth rate, resistance and tolerance to adverse conditions) on the other. Consequently, it can as well be viewed as a composite quantitative variable, a product of the interaction between the time since establishment and the growth conditions (Stankova et al. 2016). Stand mean diameter, on the other hand, is a parameter negatively correlated with stand density and therefore its value reflects the stand stocking rate. It can also be viewed as a composite quantitative variable, a product of the interaction between the growth potential of the trees, speeding up the self-thinning process when high, and stand stocking. A study by Zhang et al. (2021) revealed that the inclusion of the interaction effects of stand density and site index could significantly improve the prediction accuracy of the height-diameter model for *Larix olgensis* Henry. In addition, the modified Gaffrey's model derived here specifies the estimated tree height value according to the social status of the tree as well. Indeed, both addends of the exponent $(1-D_m/d)$ and $(1/D_m-1/d)$ additionally amplify the height estimates of trees of diameters above the stand average and reduce those of the smaller-sized trees. The model was obtained from Gaffrey's equation (1988) after replacing dominant stand height by a linear function of mean height and constraining the parameter multiplied by $(1/D_m-1/d)$ to be equal to 1. This modification removed the bias revealed by the model predecessor and produced significant pa-rameter estimates.

Gaffrey's equation and other models such us those by Sloboda et al. (1993) and Šmelko et al. (1987) originated

from a simple relationship, very popular in forest modelling and known as Schumacher function or Michailoff function (Gadow and Hui 1999). It possesses all desired features recommended by Yuancai and Parresol (2001) for functions used to model height-diameter relationships: to increase monotonically, to have an upper asymptote, and to have an inflection point. Van Laar and Akça (2007) examined and compared the model with a similar, but monotone increasing relationship without upper asymptote. The authors used both estimated functions to calculate the regression height of the tree with the arithmetic and quadratic mean diameter, the median, the diameter of the tree with the mean volume, the central area basal area tree, the mean derived from the Weise rule, for the quadratic mean of the 100 thickest trees per hectare, and those of the 10th and 90th percentile of the diameter distribution. They found that the differences between the height estimates based on both equations were almost negligible for the central values, but substantial for top height. The estimates of model efficiency and prediction errors as well as the equivalence tests conducted to validate the generalised height-diameter model derived in our study asserted its adequacy in forecasting top and dominant stand heights of Scots pine plantations in Bulgaria.

CONCLUSIONS

Considering the state-of-the-art of forest inventory in Bulgaria, our investigation pursued development of a parsimonious generalised height-diameter model for the Scots pine plantations in the country. After comparison of a number of 2-, 3- and 4- predictor candidate models, a modified form of Gaffrey's model (Gaffrey 1988) was selected. It predicts the height of a tree from the breast-height tree diameter, mean stand height and diameter, and accounts for the tree social status. The model was derived using large and representative data set and its adequacy was substantiated by verification and validation tests. The model can be broadly used, because it requires information readily available from the inventories. Its application in dominant stand height estimation is recommended.

Appendix A

Table A1. Models based on quadratic mean stand diameter (D_m) and mean stand height (H_m).

№	Model formulation	Author and source
1	$h = b_0 + b_1(d/D_m) + b_2 H_m$	Lei X, Peng C, Wang H, Zhou X, 2009. Individual height–diameter models for young black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) plantations in New Brunswick, Canada. *For Chron* 85: 43.56.
2	$\ln(h-1.3) = b_0 + b_1 \ln(d/Dm) + b_2 \ln(H_m)$	Lei X, Peng C, Wang H, Zhou X, 2009. Individual height–diameter models for young black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) plantations in New Brunswick, Canada. *For Chron* 85: 43.56.
3	$h = b_0 + b_1 H_m + b_2 D_m^{0.95} + b_3 e^{-0.08d} + b_4 H_m^3 e^{-0.08d} + b_5 D_m^3 e^{-0.08d}$	Cox F, 1994. Modelos parametrizados de altura, Informe de convenio de investigación interempresas. (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245.
4	$h = \left(1.3^{b_1} + (H_m^{b_1} - 1.3^{b_1}) \dfrac{1 - e^{-b_0 d}}{1 - e^{-b_0 D_m}} \right)^{1/b_1}$	Schnute J, 1981. A versatile growth model with statistically stable parameters. *Can J Fish Aquatic Sci* 38, 1128–1140. **After** Castedo-Dorado F, Dieguez-Aranda U, Barrio M, Sanchez M, Gadow Kv, 2006. A generalized height-diameter model including random components for radiata pine plantations in northwestern Spain. *For Ecol Manage* 229: 202-213.
5	$h = 1.3^{b_1} + (H_m^{b_1} - 1.3^{b_1}) \dfrac{1 - e^{-b_0 d}}{1 - e^{-b_0 D_m}}$	modified from 4
6	$h = 1.3 + (H_m - 1.3) e^{b_0 (1 - \frac{d}{D_m})} e^{b_1 (\frac{d}{D_m} - \frac{1}{d})}$	Sloboda VB, Gaffrey D, Matsumura N, 1993. Regionale und locale Systeme von Höhenkurven für gleichaltrige Waldbestände. *Allg Forst Jagdztg* 164: 225-228. (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245).
7	$h = 1.3 + (H_m - 1.3) e^{(b_0 + b_1 H_m + b_2 D_m)(\frac{1}{d} - \frac{d}{D_m})}$	Šmelko Š, Pánek F, Zanvit B, 1987. Matematická formulácia systému jednotných výškových kriviek rovnovekých porastov SSR. *Acta Facultatis Forestalis Zvolen* 19: 151-174. (cited in: Adamec Z, 2015. Comparison of linear mixed effects model and generalized model of the tree height-diameter relationship. *J For Sci* 61(10): 439-447).

Abbreviations: h - tree height (m), d - diameter at breast height (cm), H_m - mean stand height (m), D_m - quadratic mean stand diameter (cm), b_0, b_1, b_2, b_3, b_4, b_5- model parameters.

Table A2. Models based on dominant stand diameter (D_0) and dominant stand height (H_0).

№	Model formulation	Author and source
1	$h=1.3+(H_0-1.3)(\frac{d}{D_0})^{b_0}$	Cañadas N, Garciá C, Montero G, 1999. Relación altura-diámetro para *Pinus pinea* L. en el Sistema Central. In: Actas del Congreso de Ordenación y Gestión Sostenible de Montes, Santiago de Compostela, 4-9 October 1999. Volume I, 139-153.
2	$h=1.3+\dfrac{d}{\frac{D_0}{H_0-1.3}\,b_0(D_0-d)}$	Cañadas N, Garciá C, Montero G, 1999. Relación altura-diámetro para *Pinus pinea* L. en el Sistema Central. In: Actas del Congreso de Ordenación y Gestión Sostenible de Montes, Santiago de Compostela, 4-9 October 1999. Volume I, 139-153.
3	$h=1.3+(H_0-1.3)\,\dfrac{1-e^{b_0 d}}{1-e^{b_0 D_0}}$	Cañadas N, Garciá C, Montero G, 1999. Relación altura-diámetro para *Pinus pinea* L. en el Sistema Central. In: Actas del Congreso de Ordenación y Gestión Sostenible de Montes, Santiago de Compostela, 4-9 October 1999. Volume I, 139-153.
4	$h=1.3+(b_0(\frac{1}{d}-\frac{1}{D_0})+(\frac{1}{H_0-1.3})^{1/2})^{-2}$	Cañadas N, Garciá C, Montero G, 1999. Relación altura-diámetro para *Pinus pinea* L. en el Sistema Central. In: Actas del Congreso de Ordenación y Gestión Sostenible de Montes, Santiago de Compostela, 4-9 October 1999. Volume I, 139-153.
5	$h=1.3+(H_0-1.3)\,\dfrac{e^{b_0 d^{b_1+b_2(H_0-1.3)}}}{e^{b_0 D_0^{b_1+b_2(H_0-1.3)}}}$	Krumland BE, Wensel LC, 1988. A generalized height-diameter equation for coastal California species. *West J Appl For*, 3(4), 113-115.
6	$h=1.3+b_0 H_0^{b_1}(1-e^{-b_2 d D_0^{b_3}})^{b_4}$	*After* Sharma M, Parton J, 2007. Height–diameter equations for boreal tree species in Ontario using a mixed-effects modeling approach. *For Ecol Manage* 249(3): 187-198.
7	$h=1.3+(H_0-1.3)e^{(b_0+b_1 H_0)(1/d-1/D_0)}$	*After* Tomé, M., 1988. Modelação Do Crescimento Da Árvore Individual Em Povoamentos De *Eucalyptus globulus* Labill. (1a Rotação). Região Centro De Portugal. Ph.D. Thesis, ISA, Lisbon, 256 p. (cited in: Stankova TV, Diéguez-Aranda U, 2013. Height-diameter relationships for Scots pine plantations in Bulgaria: optimal combination of model type and application. *Ann For Res* 56 (1): 149-163).
8	$h=1.3+e^{b_0+b_1 H_0+\frac{b_2 D_0}{d+1}}$	*After* Tomé, M., 1988. Modelação Do Crescimento Da Árvore Individual Em Povoamentos De *Eucalyptus globulus* Labill. (1a Rotação). Região Centro De Portugal. Ph.D. Thesis, ISA, Lisbon, 256 p. (cited in: Stankova TV, Diéguez-Aranda U, 2013. Height-diameter relationships for Scots pine plantations in Bulgaria: optimal combination of model type and application. *Ann For Res* 56 (1): 149163).
9	$h=1.3+(H_0-1.3)(\frac{d}{D_0})^{b_1}$	Tomé, M., 1988. Modelação Do Crescimento Da Árvore Individual Em Povoamentos De *Eucalyptus globulus* Labill. (1a Rotação). Região Centro De Portugal. Ph.D. Thesis, ISA, Lisbon, 256 p. (cited in: Sánchez-González M, Cañellas I, Montero G, 2007. Generalized height-diameter and crown diameter prediction models for cork oak forests in Spain. *For Syst* 16(1): 76-88)
10	$h=1.3+(H_0-1.3)e^{b_1(1/d-1/D_0)}$	Tomé, M., 1988. Modelação Do Crescimento Da Árvore Individual Em Povoamentos De *Eucalyptus globulus* Labill. (1a Rotação). Região Centro De Portugal. Ph.D. Thesis, ISA, Lisbon, 256 p. (cited in: Sánchez-González M, Cañellas I, Montero G, 2007. Generalized height-diameter and crown diameter prediction models for cork oak forests in Spain. *For Syst* 16(1): 76-88).
11	$h=1.3+\dfrac{H_0-1.3}{1+b_1(H_0-1.3)(1/d-1/D_0)}$	Tomé, M., 1988. Modelação Do Crescimento Da Árvore Individual Em Povoamentos De *Eucalyptus globulus* Labill. (1a Rotação). Região Centro De Portugal. Ph.D. Thesis, ISA, Lisbon, 256 p. (cited in: Sánchez-González M, Cañellas I, Montero G, 2007. Generalized height-diameter and crown diameter prediction models for cork oak forests in Spain. *For Syst* 16(1): 76-88).
12	$h=1.3+(H_0-1.3)e^{b_1\frac{1-D_0}{d}+b_2(1/D_0-1/d)}$	Crecente-Campo F, Tomé M, Soares P, Diéguez-Aranda U, 2010. A generalized nonlinear mixed-effects height-diameter model for *Eucalyptus globulus* L. in northwestern Spain. *For Ecol Manage* 259: 943-952.
13	$h=b_0+H_0(1-e^{\frac{-b_1 d}{\ln D_0}})^{b_2}$	Pienaar, L.V., W.M. Harrison and J.W. Rheney. 1990. PMRC yield prediction system for slash pine plantations in the Atlantic coast flatwoods. PMRC Technical Report 1990-3. Plantation Management Research Cooperative, Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, GA, 31p. *Modified in*: Sonmez T, 2009. Generalized height-diameter models for *Picea orientalis* L. *J Environ Biol* 30: 767—772).
14	$h=H_0\dfrac{(1-e^{b_1 d})^{b_2}}{(1-e^{b_1 D_0})^{b_2}}$	Cimini D, Salvati R, 2011. Comparison of generalized nonlinear height-diameter models for *Pinus halepensis* Mill. and *Quercus cerris* L. in Sicily (Southern Italy). *Ital For Mont* 66: 395-400.
15	$h=H_0\dfrac{(1-e^{b_1 d})^{b_2+b_3 H_0}}{(1-e^{b_1 D_0})^{b_2+b_3 H_0}}$	Cimini D, Salvati R, 2011. Comparison of generalized nonlinear height-diameter models for *Pinus halepensis* Mill. and *Quercus cerris* L. in Sicily (Southern Italy). *Ital For Mont* 66: 395-400.
16	$h=H_0\dfrac{(1-e^{(b_1+b_2 H_0)d})^{b_3+b_4 H_0}}{(1-e^{(b_1+b_2 H_0)D_0})^{b_3+b_4 H_0}}$	Cimini D, Salvati R, 2011. Comparison of generalized nonlinear height-diameter models for *Pinus halepensis* Mill. and *Quercus cerris* L. in Sicily (Southern Italy). *Ital For Mont* 66: 395-400.
17	$h=(b_1+b_2 H_0)(1-e^{d(b_3+b_4 H_0)})^{b_5+b_6 H_0}$	Cimini D, Salvati R, 2011. Comparison of generalized nonlinear height-diameter models for *Pinus halepensis* Mill. and *Quercus cerris* L. in Sicily (Southern Italy). *Ital For Mont* 66: 395-400.
18	$h=1.3+\dfrac{H_0-1.3}{1-b_1(\frac{D_0}{d})^{b_2}}$	Diéguez-Aranda U, Barrio Anta M, Castedo-Dorado F, Álvarez-González JG, 2005. Relación altura-diámetro generalizada para masas de *Pinus sylvestris* L. procedentes de repoblación en el noroeste de España. *Invest Agrar: Sist Recur For* 14(2): 229-241. (cited in: Sánchez-González M, Cañellas I, Montero G, 2007. Generalized height-diameter and crown diameter prediction models for cork oak forests in Spain. *For Syst* 16(1): 76-88).

Abbreviations: h - tree height (m), d - diameter at breast height (cm), H_0 - dominant stand height (m), D_0 – dominant stand diameter (cm), b_0, b_1, b_2, b_3, b_4, b_5, b_6 - model parameters.

Table A3. Models based on quadratic mean stand diameter (D_m) and dominant stand height (H_0).

№	Model formulation	Author and source
1	$h=H_0(1+b_0e^{b_1H_0})\ (1+e^{-b_2d/H_0})$	Harrison WC, Burk TE, Beck DE, 1986. Individual tree basal area increment and total height equations for Appalachian mixed hardwoods after thinning. *South J Appl For* 10(2): 99-104.
2	$h=1.3+(H_0-1.3)e^{b_0(1/D_m-1/d)+b_1(1-D_m/d)}$	Gaffrey D, 1988. Forstamts-und bestandesindividuelles Sortimentierungs-programm als Mittel zur Planung, Aushaltung und Simulation. Diplomarbeit Forscliche Fakultät, Universität Göttingen. (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245.)
3	$h=1.3+(H_0-1.3)e^{b_0(1/D_m-1/d)}$	***After*** Diéguez-Aranda U, Castedo-Dorado F, Álvarez González JG, Rojo Alboreca A, 2006. Dynamic growth model for Scots pine (*Pinus sylvestris* L.) plantations in Galicia (north- western Spain). *Ecol Model* 191: 225-242.
4	$h=b_0H_0(1-e^{-b_1d/D_m})^{b_2}$	Pienaar, L.V., W.M. Harrison and J.W. Rheney. 1990. PMRC yield prediction system for slash pine plantations in the Atlantic coast flatwoods. PMRC Technical Report 1990-3. Plantation Management Research Cooperative, Warnell School of Forestry and Natural Resources, The University of Georgia, Athens, GA, 31 p. ***Modified in*** López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245.
5	$h=1.3+b_0H_0^{b_1}d^{b_2H_0^{b_3}}$	Gadow Kv, Hui G, 1999. Modelling Forest Development. Kluwer Academic Publishers, Dordrecht, The Netherlands, 189 p.
6	$h=1.3+(b_0+b_1H_0-b_2D_m)e^{-b_3/d}$	Mirkovich D, 1958. Normale visinske krive za chrast kitnak i bukvu v NR Srbiji. *Glasnik sumarskog faculteta* 13, Zagreb (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245).
7	$h=1.3+(b_0+b_1H_m-b_2D_m)\cdot e^{-b_3/\sqrt{d}}$	Schröder J, Álvarez González JG, 2001. Comparing the performance of generalized diameter-height equations for Maritime pine in North western Spain. *Forstwiss Centralbl* 120: 18-23.

Abbreviations: h - tree height (m), d - diameter at breast height (cm), D_m - quadratic mean stand diameter (cm), H_0 - dominant stand height (m), b_0, b_1, b_2, b_3 - model parameters.

Table A4. Models including measures of stand stocking (basal area and/or stand density) and stand-level measures of height and diameter.

№	Model formulation	Author and source
1	$h=1.3+b_0H_0^{b_1}(1-e^{-b_2d(\frac{N}{G})^{b_3}})^{b_4}$	Sharma M, Parton J, 2007. Height–diameter equations for boreal tree species in Ontario using a mixed-effects modeling approach. *For Ecol Manage* 249(3): 187-198.
2	$h=1.3+b_0G^{b_1}(1-e^{-b_2d})$	Lei X, Peng C, Wang H, Zhou X, 2009. Individual height–diameter models for young black spruce (*Picea mariana*) and jack pine (*Pinus banksiana*) plantations in New Brunswick, Canada. *Forest Chron* 85(1): 43-56. ***After*** Sharma M, Zhang SY, 2004. Height–diameter models using stand characteristics for *Pinus banksiana* and *Picea mariana*. *Scand J Forest Res* 19: 442-451.
3	$h=1.3+b_0G^{b_1}(1-e^{-b_2dN^{b_3}})^{b_4}$	Sharma M, Zhang SY, 2004. Height–diameter models using stand characteristics for *Pinus banksiana* and *Picea mariana*. *Scand J Forest Res* 19: 442-451.
4	$h=e^{b_0+b_1\ln(D_m)+b_2\ln(N)+b_3/\sqrt{d}}$	Cox F, 1994. Modelos parametrizados de altura, Informe de convenio de investigación interempresas. (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245).
5	$h=H_m(b_0+b_1H_m+b_2\dfrac{H_m}{2D_m}+b_3d+b_4\dfrac{N}{\frac{D_m(H_mD_m)}{d}})$	Cox F, 1994. Modelos parametrizados de altura, Informe de convenio de investigación interempresas. (cited in: López- Sánchez CA, Varela JG, Castedo-Dorado F, Rojo A, Rodríguez- Soalleiro R, Álvarez González JG, Sánchez-Rodríguez F, 2003. A height-diameter model for *Pinus radiata* D. Don in Galicia (Northwest Spain). *Ann For Sci* 60: 237-245).
6	$h=1.3+(b_0+b_1H_0-b_2D_m+b_3G)e^{-b_4/\sqrt{d}}$	Schröder J, Álvarez González JG, 2001. Comparing the performance of generalized diameter-height equations for Maritime pine in North western Spain. *Forstwiss Centralbl* 120: 18-23.
7	$h=1.3+b_1d^{b_2}d^{b_3}N^{b_4}G^{b_5}$	Staudhammer C, LeMay V, 2000. Height prediction equations using diameter and stand density measures. *For Chron* 76(2): 303-309.
8	$h=1.3+b_1(1-e^{b_2d^{b_3}N^{b_4}G^{b_5}})$	Staudhammer C, LeMay V, 2000. Height prediction equations using diameter and stand density measures. *For Chron* 76(2): 303-309.

Abbreviations: h - tree height (m), d - diameter at breast height (cm), H_m - mean stand height (m), D_m - quadratic mean stand diameter (cm), H_0 - dominant stand height (m), G - stand basal area ($m^2 \cdot ha^{-1}$), N - stand density (trees·ha^{-1}), b_0, b_1, b_2, b_3, b_4, b_5- model parameters.

Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

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SEEFOR SOUTH-EAST EUROPEAN FORESTRY

Physical and Fuel Properties of *Bambusa vulgaris* of Different Age Groups and Their Effect on Producing Biofuel

Kwaku Antwi [1,*], George Adu [2], Sylvia Adu [3], Joseph Appiah-Yeboah [4]

(1) Akenten Appiah - Menka University of Skills Training and Entrepreneurial Development Faculty of Technical Education, Department of Construction and Wood Technology Education, P.O Box 1277, Kumasi, AAMUSTED Avenue 12, 00000 Tanoso-Kumasi, Ghana; (2) Kumasi Technical University, Faculty of Built and Natural Environment, Department of Interior Design and Materials Technology, P. O. Box 854, Kumasi, Asafo Avenue 4, 00000 Asafo-Kumasi, Ashanti Region, Ghana; (3) Kwame Nkrumah University of Science and Technology, Faculty of Renewable Natural Resources, Department of Forest Resources Technology, Mango Road, St 35 00000 KNUST-Kumasi, Ghana (4) Mampong Technical College of Education, Department of Technical and Vocational Education, Box 31, MAMPTECH Avenue 56, 00000 Mampong – Ashanti, Ghana

* Correspondence: e-mail: kantwi@aamusted.edu.gh

ABSTRACT

Bamboo can be used to produce solid, gaseous, and liquid fuels, as well as to generate heat and biofuels for transport and electricity. The main objective of this study was to investigate the physical and fuel properties of *Bambusa vulgaris* in three ecological zones for their utilization potential for the production of biofuels. Thirty-six samples of *Bambusa vulgaris* culms from 3 ecological zones in Ghana were milled to powder to assess the physical and fuel properties. The physical properties; moisture content, high heating values, bulk density, density, and ash content were investigated. The fuel properties were based on ultimate analysis, carbon, hydrogen nitrogen, and oxygen. The mean moisture content (MC) for the green bamboo ranged from 68.8% (dead culm) to 148% (mature culm) and 168% (juvenile culm). The MC for dried samples ranged from 9.09 to 13.06%. The results showed that the % MC of the samples increased with decreasing values of high heating and ash content. The mean density of the matured bamboo culms varied from 616.84 to 641.68 kg·m⁻³ and dead bamboo culms from 609.01 to 632.72 kg·m⁻³. Marginal reduction in density was observed in dead bamboo culms across the three ecological zones as compared to the mature bamboo culms. This implies that when *B. vu*lgaris overgrows, its density decreases. High heating values ranged from 16.12 to 18.14 MJ·kg⁻¹. Bulk density determines the transportation and storage of biomass; it ranged from 0.12 to 0.52 g·m⁻³. The ash contents were within the threshold (≥3%) of European standard. The mean values for the ultimate analysis of carbon (48.46 to 53.31%), hydrogen (5.60 to 6.56%), nitrogen (0.58 to 0.61%), and oxygen (39.73 to 41.35) were higher. Fuel properties of *Bambusa vulgaris* are comparable to some wood types, lower to denser wood types, but higher than most of the herbaceous energy crops and agricultural residues. This means that *Bambusa vulgaris* may be a good feedstock for the production of bioenergy in terms of heat, charcoal, biogas, bio-power, and transportation fuel.

Keywords: Bamboo; biomass; biofuel; high heating value

INTRODUCTION

The world demand for petroleum-based fuels has increased as a result of increasing industrialization and motorization (Agarwal 2007). Resources like gasoline, coal, natural gas, diesel and other products derived from fossil fuels are non-renewable (Brown 2003) because they cannot be regenerated once used. Many researchers reported that the burning of fossil fuels emits extensive greenhouse gases, such as carbon dioxide, into the atmosphere, which are the sources of some other severe environmental problems (Davis and Caldeira 2010, Street and Yu 2011). The intensive use of fossil fuels and other natural resources accumulate greenhouse gases which cause heat over the Earth's surface, resulting in changes in temperature and other climatic processes (Koutsoyiannis et al. 2009). These emissions among others have brought about climate change such as global warming, flooding (World Bank 2009), droughts, famines, water shortages, extreme heat (Koutsoyiannis et al. 2009, Riché et al. 2009), and desertification (World Bank 2009).

Global wood charcoal production rose from 17.3 to 53.1 million tons between 1964 and 2014. Sixty-one percent of the present global production of charcoal occurs in Africa to satisfy the cooking demand from urban households (Doggart and Meshack 2017). World Health Organization (WHO) revealed that over 75 percent of rural households and around 20 percent of urban households depend primarily on wood fuel for cooking in low- and medium-income countries (WHO 2016). A shift from fossil fuel-based energies to dependence on renewable energy had been discussed by many researchers (Demirbas and Arin 2002, Prins 2005, White 2010). Several researchers have proposed the use of these renewable energies as substitutes for fossil fuels; solar, wind, hydro, ocean thermal energy, and biomass. Among the renewable energy sources mentioned above, biomass is the only carbon-based sustainable energy (Sathre and O'Connor 2010). Biomass can be burned to produce heat and electricity; changed to gas-like fuels such as methane, hydrogen, and carbon monoxide, or changed to liquid fuel. Liquid fuels, also called biofuels, include mainly two forms of alcohol: ethanol and methanol (Demirbas 2010).

Wood and energy crops were the main biomass substrate for the production of biofuels (Demirbas and Arin 2002, Filho and Badr 2004), agricultural and forestry residues, animal wastes, (Demirbas and Arin 2002, Bridgwater 2004) municipal solid wastes, manufacturing wastes, and vegetable oils (Filho and Badr 2004). Trees and crops can always be grown and waste will always exist (The NEED Project 2011). By nature, biomass materials vary depending on geographical location, variety, climate conditions, and harvest methods (Clarke and Preto 2011). The demand for biofuel was insatiable; as a result, many researchers around the world have delved into using lignocellulose materials such as wood, crops, and agricultural wastes to produce biofuels (IEA 2010). Between 2000 and 2010, global biofuel production grew from 16 billion liters to 100 billion liters (IEA 2011). However, frequent use of woody biomass for bioenergy (electricity, gas, or heat) production is expected to have some ripple effects in the forest and agriculture sectors. Increased use of the mill and forest residues for bioenergy will likely decrease the production of oriented strand boards, bark mulch recycles soil nutrients, and improve micro-climate site conditions (White 2010). Countries like Brazil, Canada, and the USA are using sugar cane, wheat, corn, or soya respectively to produce bioethanol (Koh and Wilcove 2008). Food crops used for biofuels have adverse effects on the food supply to humans, poultry, and farm animals and increase food prices (Sun and Cheng 2002). Bamboo was seen by many researchers as the most promising energy source to mitigate greenhouse gas emissions (Demirbas 2010, Preto 2010). Bamboo has a very wide variety of energy needs, including generating electricity (Demirbas 2010, Preto 2010), high heating homes (Preto 2010), fuelling vehicles (Bain 2010), and providing process heat for industrial facilities (Preto 2010). Bamboo is a sustainable, fast-growing plant that takes 3 to 4 years to mature (INBAR and BARADEP 2003). *Bambusa vulgaris* is the most common type of bamboo, which covers about 95% of the total growing area in the southern part of Ghana (Oteng-Amoako et al. 2005).

The knowledge of utilizing bamboo for the production of biofuels is increasing, but most of the studies dealt with only studied mature culms (Choy et al. 2005), neglecting the potency of the shoot, young and dead culms, branches, and leaves. Scurlock et al. (2000) studied nine bamboo ages from 1 to 5 years, i.e. juvenile and mature culms. However, they did not consider ecological zones, shoots, over-grown or dead culms for fuel properties. Again, little is known about the characteristics of bamboo as a feedstock for the production of biofuel in West Africa. This study aims to determine the physical and fuel properties of *Bambusa vulgaris* Schrader ex Wend land *var. vulgaris* (Bamboo) age groups: shoots, juvenile, mature, dead, or overgrown. The tested hypothesis of the study is to determine the physical and fuel properties such as density, bulk density, and high heating value of *Bambusa vulgaris* age group culms. The results imply utilizing these bamboo parts for energy production.

MATERIALS AND METHODS

The Study Areas

The samples of bamboo were collected in their natural stands from three ecological zones in Ghana. These areas were dry semi-deciduous zone (DSD), moist semi-deciduous (MSD), and moist evergreen deciduous (MED). The DSD zone is located in the transitional zone between Ghana's forest and Savanna regions (FAO 2005). The area covered a forest near Techiman in the Brong East Region of Ghana, which is between 7°35' N latitude and 1°48' W longitude. The mean annual rainfall ranges from 1300 to 1400 mm (Obiri and Oteng-Amoako 2007). The highest annual temperature is 33°C (Duku et al. 2011). The next area of study was Owabi forest reserve, near Kumasi, which is positioned between 6°46' N latitude and 2°3' W longitude in Ashanti Region. The forest is located in the moist semi-deciduous (MSD) forest zone (Hall and Swaine 1981). The average annual rainfall ranges from 1400 to 1750 mm (Obiri and Oteng-Amoako 2007) and the highest annual temperature is 31°C (Duku et al. 2011). The last investigated area was Bonsa River Forest Reserve in the moist evergreen – deciduous (MED) zone. The reserve is located between the coordinates of 5°15' latitude and 2°45' W longitude in the Western region of Ghana. The mean annual rainfall ranges from 1700 to 2800 mm (Obiri and Oteng-Amoako 2007)**.**

Preparation of the Samples

Four age groups of *Bambusa vulgaris,* shoot, juvenile, mature and dead culms, were used for the experiment. The juvenile, mature and dead bamboos were measured at 300 mm intervals each. Mature branches (green and dry) and leaves (green and dry) were characterized in terms of moisture content, basic density, ultimate composition, high heating values, and ash content. The samples were oven-dried at 103±2°C and pulverized to fine powder by using a Wiley mill (Scurlock et al. 2000, Templeton et al. 2009). The milled samples were mixed toughly to obtain homogenization in a sieve shaker to pass through a 425 μm mesh sieve but retained on a 250 μm mesh sieve. The powder obtained was oven-dried again for the fuel analysis.

Determining the Moisture Content

Two grams of each powdered sample were weighed on an analytical balance and placed in different crucibles. The crucibles were then labelled and placed in the oven which was kept at a temperature of 105˚C. Each crucible and sample were taken out of the oven regularly and weighed. Gradual decreases in weight were observed and the drying and weighing continued until there was no observed change in the weights after several weighing. The calculation is as follows:

$$\% \text{ Moisture Content (MC)} = \left(\frac{M - M_o}{M_o}\right) \times 100 \quad (1)$$

where M is the mass of the test piece before drying, and M_o is the mass of the test piece after drying.

Determining Bulk Density Measurement

Bulk density is also called volumetric or apparent density. This is where the properties of powders, granules, and other divided solids are compacted or tapped in volume. Bulk density increases with compaction and tends to increase with depth. The measurement of bulk density was based on ASTM E873 (2013). The powdered bamboo sample was sieved to a 60-mesh screen (250 microns). The fresh sample was weighed (W_1, dry at 105°C for 2 days), and weighed again (W_2). The calculation is as follows:

$$\text{Bulk density} = W_2/V \text{ (g} \cdot \text{cm}^{-3}) \quad (2)$$

$$\text{Water content (Theta)} = (W_1 - W_2)/V \text{ (cm}^3 \cdot \text{cm}^{-3}) \quad (3)$$

Determining Dry Density

The density of solid bamboo was determined by using ISO 3131 (1975) standard. The dry density is the ratio of the density of a test specimen to its volume. The density was used to report the natural moisture content of the test specimen. Here the mass is taken as the oven-dried mass and only the volume is taken at the natural moisture content of the specimen. The density of each test sample is calculated by using Equation 4:

$$D = m/v \ (10^6) \quad (4)$$

where D is density in kg·m^{-3}, m is the oven-dried mass in grams of the test specimens, and V is the oven-dried volume of the test specimens in mm^3.

Determining High Heating Value (HHV)

The powdered bamboo sample was pelleted and oven-dried to constant weight at 80°C. It was then burned in an oxygen bomb calorimeter calibrated by burning benzoic acid in the same way as the sample (Technical Support Document 2007). The calculations were as follows:

Heat capacity of the system

$$\text{Ccal} = Q / \Delta T \cdot c \quad (5)$$

where Ccal is heat capacity of the calorimeter, Q is heat capacity of the object, ΔT is change in temperature and c is specific heat capacity;

$$Q = m/\Delta H \quad (6)$$

where ΔH is molar combustion enthalpy of benzoic acid = − 3231.5 kJ/mol and m is molar mass of Benzoic acid.

Determining the Percentage (%) Weight of Ash Content in the Bamboo Age Groups

The ash content of the bamboo was determined by ASTM D 1102 (2008). Two grams of the powdered bamboo sample were oven-dried in an electric furnace at a temperature of 600˚C for four hours. The percentage of the ash content was calculated as follows:

$$\text{Ash content (\%)} = \frac{M_{ash}}{M_{oven\text{-}dry}} \times 100 \quad (7)$$

where M_{ash} is the mass of the ash and $M_{oven\text{-}dry}$ is the mass of the oven-dried sample.

Determining the Percentage (%) Weight of Carbon

Carbon in the bamboo was determined by using Walkley – black wet oxidation method (Nelson and Sommers 1982). The percentage of carbon (C) was calculated as:

$$\% \text{ C} = \frac{N \times (Vbl\text{-}Vs) \times 0.003 \times 1.33 \times 100}{g} \quad (8)$$

where N is Normality of ferrous sulphate = 0.5 N, Vbl is liter value of the blank solution, Vs is liter value of sample solution, g is mass of sample taken, 0.003 is milliliter equivalent weight of C in grams (12/4000), 1.33 is correction factor used to convert the wet combustion C value to true C value since the wet combustion method is about 75% efficient in estimating the C value (100/75).

Determining the Percentage (%) Weight of Hydrogen

The hydrogen content was determined using the exchangeable acidity titrimetric method (Mclean 1965). The following calculation was used:

$$\frac{V*0.05*100}{W} = V* \quad (9)$$

where V is titre volume of NaOH used (ml); normality of NaOH = 0.05N and W is weight of sample used (1.0 g).

The Percentage (%) of Nitrogen

The Kjeldahl method was used to determine nitrogen in chemical substances (Motsara and Roy 2008). The weight of N was calculated as: 14 g of contained in one mile-equivalent weight of NH_3:

$$\text{The weight of N in the sample} = \frac{14 \times (A\text{-}B) \times \text{concentration of acid}}{1000} \quad (10)$$

where A is volume of standard HCl used in the sample titration and B is volume of standard Cl used in the blank titration.

The percentage of nitrogen in the sample is calculated as:

$$\% \text{ N} = \frac{14 \times (A\text{-}B) \times \text{concentration of acid}}{1000} \quad (11)$$

Determining Nitrogen (Kjeldahl Method)

One gram of the shell/nib was weighed and transferred into a 500 ml digestion flask and 30 ml of H_2SO_4 was added.

The digestion flask with the mixture was heated in the DK20 high heating digester block starting at a temperature of 80°C and then the temperature was raised to 350°C. The content of the digestion flasks was heated until the volume was reduced to 3 to 4 ml. The content of the digestion flasks was cooled and the volume made up to 100 ml in volumetric flasks. The volumetric flasks were labelled accordingly. Ten millilitres of sample digest were transferred using a pipette into a Kjeldahl distillation apparatus after the addition of 20 ml of 40% NaOH. The distillate was collected over 10 ml of 4% boric acid and three drops of mixed indicator in a 250 ml conical flask for 5 minutes. The presence of nitrogen gave a light blue colour. 200 ml of the distillate was titrated with 0.1 N HCl till the colour changed from light blue to grey and suddenly flashed to pink. A blank was carried out with the solution sample. The weight of N was calculated as 14 g of N contained in one mile-equivalent weight of NH_3.

RESULTS

Moisture Content

In green conditions, the moisture content of *Bambusa vulgaris* varies with the age and height of the culm and the geographical location of the bamboo (Figure 1). The moisture content decreases from the shoot to the over-grown (dead) culm and also from the bottom or base of the culm to the top in all three ecological zones. Among the bamboo culms sampled from the three ecological zones, those from the moist evergreen ecological zone had the highest average moisture content of 157% (juvenile), 148% (mature), and 68.8% (dead).

One-way ANOVA test showed that at 5% level of significance the average moisture content of the shoot (F-statistic=11.04, p-value=0.010), juvenile culms (F-statistic=5.58, p-value=0.043), mature culms (F-statistic=22.03, p-value=0.002) and over-mature culms (F-statistic=48.62, p-value=0.001) sampled from the three ecological zones differed significantly. The coefficient of variation of the shoot culms, juvenile culms, mature culms, and the over-mature culms were 5%, 6%, 10%, and 11% respectively.

The Percentage Dry Moisture Content of *Bambusa vulgaris* Across the Three Ecological Zones

The mean moisture content of the dry bamboo samples increased from dry semi-deciduous zone to moist evergreen deciduous zone among the age groups. Table 10 shows the moisture content (dry bamboo); the shoots ranged from 9.09% to 9.19%, juvenile scored 13.24% to 13.31%, mature culm obtained 12.93% to 13.06% and dead culm recorded 11.02% to 11.06%. It could be observed from Table 1 that

Figure 1. Variation of moisture content with bamboo age groups: **a)** Dry semi-deciduous; **b)** Moist semi-deciduous; **c)** Moist evergreen; **d)** Variation of moisture content with bamboo type.

there were statistically significant differences among the shoot, matured and the dead culm mean diameters for all the ecological zones. The test for juvenile shows positive results for shoot (p-value=0.055, juvenile (p-value<0.000), matured (p=0.002) and dead samples (p-value<0.000).

The Density of *Bambusa vulgaris* Across the Three Ecological Zones

Mature culms of the green *Bambusa vulgaris* have marginal increases in the density of the samples from the three ecological zones, ranging from 616.84 kg·m⁻³ (DSD) to 628.12 kg·m⁻³ (MSD), and 641.68 kg·m⁻³ (MED). A marginal decrease in density was observed for dead bamboos across the three ecological zones (Table 2). The mean values for the dead culm range from 609.01 kg·m⁻³ (DSD) to 620.21 kg·m⁻³ (MSD) and 635.72 kg·m⁻³ (MED).

Bulk Density of the *Bambusa vulgaris* Across the Three Ecological Zones

The mean bulk density exhibited a more definite pattern of variation within and between all the zones (Table 3). The mean values increased from dry semi-deciduous, moist semi-deciduous to the moist evergreen zone. The mean bulk density of shoot ranged from 0.13 to 0.17 g·m⁻³, juvenile from 0.17 to 0.28 g·m⁻³, mature ranged from 0.28 to 0.32 g·m⁻³ and dead recorded 0.32 to 0.52 g·m⁻³. On the whole, the samples from moist evergreen recorded the highest values. On the contrary, the samples from dry semi-deciduous got the lowest values. The mean values of the juvenile and dead culms were statistically significant.

The High Heating Values of the *Bambusa vulgaris* Across the Three Ecological Zones

The average high heating value ranged from 16.12 to 18.14 MJ·kg⁻¹. The results showed a consistent rise of values from MED upstream to MSD in the middle belt, then to DSD transitional zone between the forest and savanna regions. It could be observed from Table 4 that the highest average high heating value amongst the culms of the three zones was recorded at the dead culms (18.14 MJ·kg⁻¹) from dry semi-deciduous (DSD).

Table 1. Mean and standard deviations on the percentage moisture content (dry) of *B. vulgaris* age groups at different ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	9.09± 0.074	9.19± 0.006	9.19± 0.010	4.868	0.055
Juvenile	13.24± 0.006	13.27± 0.006	13.31 ± 0.006	94.333	0.000
Mature	12.93± 0.025	13.04±0.031	13.06± 0.030	19.257	0.002
Dead	11.02± 11.057	11.06± 0.006	11.05± 0.006	55.500	0.000

Table 2. Mean and standard deviations on density (kg·m⁻³) of the shoot, juvenile, mature and dead culms of *B. vulgaris* at different ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	300.77a± 5.74	303.10±7.97	313.20± 13.16	6.228	0.034
Juvenile	464.78 ± 10.43	466.62±15.27	472.42 ±10.54	0.342	0.723
Mature	616.84 ± 15.91	628.12 ±5.27	641.68 ±14.50	0.222	0.808
Dead	609.01 ± 10.67	620.21±13.74	635.72 ± 13.44	2.630	0.151

Table 3. Bulk density (g·m⁻³) of the shoot, juvenile, mature and dead culms of *B. vulgaris* at different ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	0.13± 0.031	0.16± 0.010	0.17± 0.025	2.480	0.164
Juvenile	0.17± 0.045	0.21± 0.036	0.28± 0.045	5.832	0.039
Mature	0.28± 0.030	0.24± 0.044	0.32± 0.030	3.892	0.082
Dead	0.32± 0.036	0.38± 0.032	0.52± 0.021	32.783	0.001

Table 4. High heating values (MJ·kg⁻¹) of the shoot, juvenile, mature and dead culms of *B. vulgaris* at different ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	16.59a±0.79*	16.19ab±0.09	16.12b±0.10	4.208	0.022
Juvenile	17.61a ± 0.57	17.54 b± 0.09	17.30a ± 1.17	7.222	0.002
Mature	17.74 a± 0.42	17.12b± 0.71	17.08c ± 0.08	9.848	0.001
Dead	18.14a ± 0.66	17.50b ± 0.72	17.29 a± 0.10	66.372	0.001

*Mean standard deviation

Means in the row with different letters indicate a significant difference at the 5 percent probability. Significant differences were found in the mature and dead bamboo culms sampled from the three zones in respect to high heating values. Culms sampled from both the dry semi-deciduous and moist evergreen had significantly higher high heating values than those from the moist semi-deciduous zone.

The Percentage (%) of Ash Content of the *Bambusa vulgaris* Samples

The values of the mean ash contents across the three ecological zones are presented in Table 5. The shoot exhibited values ranging from 1.51% (moist evergreen) to 1.72% (moist semi-deciduous zone). The average weight of ash in the juvenile samples was from 1.71% (moist evergreen) to 2.01% (moist semi-deciduous zone). The mature samples ranged from 0.93% (moist evergreen) to 1.83% (dry semi-deciduous zone). The values of the dead bamboo samples recorded were as follows: the lowest average value recorded in the moist semi-deciduous zone

was 1.15% and the highest was 2.17% located in the moist evergreen zone.

Significant differences were found for the shoot (F=7.892, p=0.021) and the mature (F=2.318, p= 0.002) sampled from the three zones in respect of ash content.

Percentage (%) Weight of Carbon Concentrations in *Bambusa vulgaris* Across the Three Zones

The mean highest carbon values rose from the dry semi-deciduous to the moist evergreen zone. The mean shoot varied a little from 49.74% in moist evergreen, 49.67% in moist semi-deciduous, and 48.46% in dry semi-deciduous zone (Table 6). The percentages of the juvenile culms were generally higher at moist evergreen (53.31%), dry semi-deciduous recorded 50.10%, while moist semi-deciduous zone had 52.24%. The mature culms obtained the following values: 48.58% in the dry semi-deciduous region, 50.84% in moist evergreen, and 52.82% in the moist semi-deciduous zone. Meanwhile, the dead bamboo culms have the following values: 52.14% for dry semi-deciduous, 52.75% for moist semi-deciduous, and 53.01% for moist evergreen zone.

Table 5. Variation of ash content (mean ±SD) with bamboo type and ecological zone (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	1.59 ± 0.50	1.72 ± 0.12	1.51 ± 0.06	7.892	0.021
Juvenile	1.79 ± 0.01	2.01 ± 0.18	1.71 ± 0.11	2.073	0.207
Mature	1.83 ± 0.64	1.52 ± 0.64	0.93 ± 0.15	2.318	0.002
Dead	1.98 ± 0.27	1.54 ± 0.54	2.11 ± 0.06	2.177	0.195

Table 6. The mean percentage of the carbon content of bamboo types and ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	48.46±2.42	49.67±0.19	49.74±0.22	0.574	0.591
Juvenile	50.10±1.44	52.24±0.17	53.31±2.78	1.566	0.284
Mature	48.58±1.26	50.84±1.81	52.82±0.10	5.993	0.037
Dead	52.14±1.64	52.75±1.93	53.01±0.54	9.253	0.015
Foliage	48.92±1.48	48.16 ± 2.09	52.16 ± 0.54	0.007	0.939
Branches	45.10± 0.59	36.56± 1.16	46.58 ± 1.17	0.163	0.901

Table 6 shows the one-way ANOVA test for the mean carbon content of *Bambusa vulgaris* in three ecological zones. Only the mature (F=5.993, p=0.037) and the dead (F=9.253, p=0.015) culms were statistically significant.

Percentage (%) Weight of Hydrogen Content in *Bambusa vulgaris* Across the Three Ecological Zones

From the results in Table 7, shoot recorded the mean values of 6.27% (dry semi-deciduous zone) to 7.04% (moist evergreen zone). The mean percentage weight of hydrogen in juvenile samples decreases marginally from dry semi-deciduous (6.17%) to moist evergreen zone (6.13%). The lowest mean percentage of hydrogen (6.34%) was recorded in the dry semi-deciduous zone and the moist semi-deciduous zone recorded the highest value of 6.56%. Meanwhile, the average hydrogen weight of dead bamboo samples ranged from 5.60% in dry semi-deciduous to 6.22% in the moist evergreen zone. The hydrogen level rose from a dry semi-deciduous to a moist evergreen zone. The highest value was recorded at the shoot (7.04%) from moist evergreen and the lowest was found in the dead (5.60%) located in a dry semi-deciduous zone.

Table 7 shows a one-way ANOVA test for the mean percentage hydrogen content in *Bambusa vulgaris* across three zones in Ghana. Only the shoot was statistically significant (F=15.571). Nonetheless, there were no significant differences among the groups.

Percentage (%) Weight of Nitrogen Concentrations in *Bambusa vulgaris* Across the Three Ecological Zones

The shoot from the moist evergreen zone recorded the lowest mean nitrogen content at (0.65%) and the highest at 2.52% in the dry semi-deciduous zone (Table 8). Juvenile samples from moist semi-deciduous recorded the lowest value of nitrogen of 0.61% and dry semi-deciduous recorded 0.79%. The nitrogen concentration in juveniles was higher than that of the rest of the culms. The percentage weight of nitrogen for mature bamboo varies from 0.58% (moist evergreen) to 0.61% (dry semi-deciduous), and the dead samples ranged from 0.32% (moist semi-deciduous) to 0.58% (moist evergreen).

There were significant effects of the amount of nitrogen on shoots (F=17.577, p<0.05) and the dead culm (F=9.056, p<0.05) of the bamboo.

The Mean Percentage Weight of Oxygen by Calculation

The mean percentage value of oxygen and standard deviation of the shoot was 40.20±2.45, the juvenile was 39.43±1.79, mature was 41.04±2.43 and dead culm was 41.35±4.05.

Fuel Characteristics of *Bambusa vulgaris* Across the Three Ecological Zones

The fuel characteristics of *Bambusa vulgaris* in terms of ultimate analysis, basic density, moisture content, and high heating values were investigated (Table 9).

Table 7. The mean percentage of the hydrogen content of bamboo types and ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	6.27±0.23	6.39±0.09	7.04±0.16	15.571	0.004
Juvenile	6.17±0.10	6.15±0.15	6.13±0.27	0.020	0.980
Mature	6.34±0.34	6.56±0.36	6.52±0.26	0.299	0.752
Dead	5.60 ± 0.51	6.20±0.51	6.22±0.02	2.028	0.212

Table 8. Percentage of the nitrogen content of bamboo types and ecological zones (DSD - dry semi-deciduous zone, MSD - moist semi-deciduous, and MED - moist evergreen deciduous).

Items	Ecological zone			ANOVA	
	DSD	MSD	MED	F-value	p-value
Shoot	2.52±0.65	0.93±0.24	0.65±0.18	17.577	0.003
Juvenile	0.79±0.06	0.61±0.05	0.63±0.12	4.611	0.061
Mature	0.61±0.09	0.61±0.34	0.58±0.05	0.020	0.980
Dead	0.48 ± 0.08	0.32±0.06	0.58±0.05	9.056	0.015

Table 9. Fuel characteristics of *Bambusa vulgaris*.

	Ultimate analysis (%)				Ash (%)	Density (kg·m⁻³)	BD (g·m⁻³)	HHV (MJ·kg⁻¹)
	C	H	N	O				
Shoot	49.29±1.50	6.56±0.38	1.37±0.90	40.20±2.45	1.30±2.52	413±13.16	0.16±0.01	16.31±0.49
Juvenile	51.38±2.18	6.15±0.18	0.67±0.18	39.43±1.79	1.81±0.99	666±10.54	0.22±0.04	17.19±0.86
Mature	50.75±2.15	6.47±0.33	0.17±0.03	41.04±2.43	1.41±0.70	722±14.50	0.28±0.03	16.98±0.82
Dead	49.97±3.24	6.06±0.37	0.14±0.02	41.35±4.05	1.88±1.35	715±13.40	0.40±0.09	16.59±1.14

C – carbon, H - hydrogen, N - nitrogen O - oxygen, BD - basic density, HHV - high heating value

The mature culms exhibited the highest fuel yield, followed by dead culms in all the zones. The yardstick was based on positive attributes such as large carbon, hydrogen, high heating value, and high basic density. These were followed by the relatively low amount of moisture content, oxygen, nitrogen, and ash content which seem to decrease the fuel content in a substrate. The third and the fourth age groups were shoots and juvenile culms all based on the positive and negative attributes of fuel properties.

Relationships Among the Fuel Properties of *Bambusa vulgaris* Culms or Stems

Combustion analysis is part of a process intended to improve fuel economy, reduce undesirable exhaust emissions and improve the safety of fuel-burning equipment (TSI Incorporated 2004). The negative effects of some fuel properties will depend on the simultaneous presence of others. For example, the higher the moisture contents of fuel, the lower the high heating values (Montano 2014).

Table 10 shows that the correlation between the high heating value and hydrogen was very strong and positive (r=0.755). The relationship between high heating value and carbon was significant (r=0.724 and p=0.05). There was an intermediate negative correlation between the high heating value and the ash content (r=-0.603; p>0.05). There was a negative correlation between ash content and the carbon content (r=-0.872; p>0.05).

The relationship between moisture content of wet and dried bamboo have strong positive correlation (r=0.727; p>0.05). There was a positive relationship between moisture content (dry basis) and the high heating value (r=0.059 and p>0.05). However, % moisture content of the wet bamboo is negatively related to high heating value (r=-0.188; p 0.05). There was negative correlation between moisture content (wet) and the ash content (r=-0.064 and p>0.05), whilst the dry bamboo is positively related to ash content (r=0.289 and p>0.05). Nitrogen is negatively related to basic density (r=-0.604 and p=0.05). There was a positive correlation between moisture content (wet bamboo) and basic density (r=0.145 and p>0.05).

DISCUSSIONS

Moisture Content in *Bambusa vulgaris* Across the Three Ecological Zones in Ghana

Moisture content varies from the bottom to the top of the fresh bamboo samples. The moisture content from the moist evergreen forest exhibited higher values than those from moist semi-deciduous and dry semi-deciduous zones in Ghana. The average moisture content of the fresh bamboo ranges from 52.43 (dead or over-mature) to 168% of the bamboo culms.

The higher the moisture contents of bamboo samples, the lower the high heating values. Efficiency is reduced because large parts of the energy available in the bamboo itself were used to heat up and evaporate this moisture. One way to increase efficiency would be to dry the fuel on-site. Higher moisture content increases both the cost of production and transportation of biofuel feedstock. High heating bamboos with more moisture content produce more smoke, which contains more volatile materials. The volatiles consist of minor and heavy metals such as arsenic, lead and sulphur, which are injurious to human health. The more moisture in the bamboo the less ash is produced. Probably most of the minerals and fixed carbon in the bamboo were evaporated in the form of smoke and steam. On the contrary, the lower the moisture content is, the more ash is produced. However, dense dried bamboo produces more low ash content than less dense bamboo.

Bulk Density of *Bambusa vulgaris* Across the Three Ecological Zones

The bulk density is in line with the research conducted by earlier researchers (0.13 to 0.52 kg·m^{-3}) on bamboo and some seasoned or dried wood such as: Alder 0.4 to 0.7; Afromosia 0.71; Agba 0.65; Apple 0.65 to 0.85; Ash, white 0.54; Ash, black 0.54; Ash, European 0.71; Bamboo 0.30 to 0.40; Mahogany, Africa 0.50 to 0.85; Teak, Africa, 0.98; Utile 0.66; Walnut 0.65 to 0.7 (Engineers' Toolbox n.d). Qisheng et al. (2002) also reported bulk density values from 0.40 to 0.90 kg·m^{-3} of *Phyllostachys pubescence*.

Table 10. Pearson's correlation test (p<0.01 and 0.05) among *Bambusa vulgaris,* ash content, basic density, bulk density, moisture content (wet), moisture content (dry), and ultimate elemental properties.

Culm/Stem	Ash (%wt)	High heating val. (MJ·kg^{-1})	Basic density (kg·m^{-1})	Bulk density (kg·m^{-1})	MC wet (%wt)	MC dry (%wt)	Carbon (%wt)	Hydrogen (%wt)
High heating val.	-.603*							
Basic density	-.305	.154						
Bulk density	-.354	.149	.362*					
MC wet %wt	-.064	-.239	-.527	-.173				
MC dry %wt	.289	.059*	-.604	-.096	.727*			
Carbon %wt	-.0872*	-.724*	.032	.119	.260	.078		
Hydrogen %wt	-.154	.755*	-.264	-.025	.429	.428	-.049	
Nitrogen %wt	.149	-.228	-.604*	-.349	.368	.253	-.052	.072

*Correlation is significant at the 0.05 level (2-tailed)

The Density of *Bambusa vulgaris* Across the Ecological Zones

The mean density of matured *Bambusa vulgaris* samples ranged from 616.84 kg·m⁻³ (DSD), to 628.12 kg·m⁻³ (MSD), and 641.68 kg·m⁻³ (MED). The results were in line with the results recorded by former authors in Ghana. For example, the basic density values ranging from 371.35 kg·m⁻³ to 684.00 kg·m⁻³ were reported by Tekpetey (2011). Ebanyenle and Oteng-Amoako (2007) recorded the basic density of *Bambusa vulgaris* in Ghana as 577.00 kg·m⁻³ in wet evergreen forest type and 684.00 kg·m⁻³ in moist semi-deciduous forest type. The data were collected in the rainy season.

The High Heating Values of *Bambusa vulgaris* Across the Three Ecological Zones

The average high heating values for the three zones were 16.12 MJ·kg⁻¹ (juvenile) to 18.14 MJ·kg⁻¹ (dead culm). It could be observed that the high heating value at the shoot and juvenile ages of bamboo in the net high heating value of bamboo is comparable to or higher than other wood species like the beach, spruce, eucalyptus, and poplars, which range from 16.02 to 18.25 MJ·kg⁻¹ (Amoah and Cremer 2017).

Bamboo has low ash content, low alkali index, and lower high heating value. However, the high heating values of bamboo are higher than most agricultural residues, grasses, and straws. Meanwhile, it is lower than many woody biomasses (Scurlock et al. 2000).

The Ash Content in *Bambusa vulgaris* Across the Three Ecological Zones

The values recorded for the ash concentrations of the bamboo culms or stems range from 0.93 to 2.11%. The results obtained were similar to that reported by previous researchers. For example, Ota (1976) reported that bamboo ash ranged from 1.7 to 5.0%. Scurlock et al. (2000) also reported considerable variation of less than 1% for three *Phyllostachys* of bamboo investigated. The shoots were within the range of 1.91% to 4.99% of five other bamboo species reported by Dannenmann et al. (2007). Ratner (2011) studied the proximate analysis of wood chips and compared it with that of coal. The wood ash was 2.24 wt %, while coal was 10.6 wt %. Ash content from switchgrass (dry basis) ranged from 4.4 wt % to 9.2 wt % (Benson and Laumb 2010).

Carbon Concentrations in *Bambusa vulgaris*

The mean percentage of carbon concentrations in mature culm varied from 48.46 to 53.31%. The findings fell within the range of 48.5% to 50% on a dry basis for bamboo (Choy et al. 2005), 45 to 55% (Ganesh 2003, Vessia 2006), and carbon contents in three bamboos based on their age (1 year to 5 years) ranged from 51.39 to 51.84 (Scurlock et al. 1999). Higher carbon content leads to a higher high heating value (Clarke and Preto 2011).

Hydrogen Concentrations in *Bambusa vulgaris*

The mean percentages of hydrogen recorded in the bamboo culms of the age groups were between 5.60 and 6.56%. These were similar to other researchers who reported that the hydrogen content of bamboo culms varies from 4.8 to 6.7% (Ganesh 2003), 6% (Jenkins 1998) and 6.0 to 6.5% (Choy et al. 2005). However, Scurlock et al. (1999) recorded the hydrogen content in three bamboos based on their age as 1 year (4.90 to 5.21%), 2 years (5.00 to 5.29%), and 4 to 5 years (4.51 to 5.40%). Higher hydrogen content leads to a higher high heating value (Clarke and Preto 2011). Hydrogen is a reducing gas and the cracking of biomass in the presence of hydrogen can reduce the oxygen content for example in bio-oil (Zhou et al. 2013).

Nitrogen Concentrations in *Bambusa vulgaris* Across the Three Ecological Zones

Nitrogen also had a mean percentage weight between 0.32 and 2.52%. The percentage of 2.52% was recorded in the shoot. The shoot is not recommended for fuel production because its values were above the threshold set by the European standard EN 15104 (2011) according to which nitrogen should be above 1% in biomass for fuel production. Apart from the shoot, the other samples were similar to the earlier studies; from 0.2% to more than 1% (Jenkins et al. 1998); 0.4 to 1.3% (Ganesh 2003), and 0.3% (Choy et al. 2005). The results of experiments by Scurlock et al. (1999) show that the nitrogen contents in three bamboos based on their age were 1 year (0.4 to 0.59%), 2 years (0.29 to 0.6%), and 4 to 5 years (0.21 to 0.38%). Oxides of nitrogen (NOx) cause ozone, smog, and respiratory problems. Wood and fuel oil combustion have similar levels of NOx emissions (Maker 2004). Nitrogen in fuel feedstock is responsible for most nitrogen oxide (NOx) emissions produced from biomass combustion. Lower nitrogen content in the fuel should lead to lower NOx emissions (Clarke and Preto 2011).

The Relationships Between the Physical and Fuel Properties of the Bamboo (Bambusa vulgaris)

The Relationship Between Wet and Dry Moisture Content

The relationship between wet moisture content and dry moisture content has a strong positive correlation, r=0.609; p>0.05. The moisture content of fresh bamboo increases the weight of the bamboo. The wetter the bamboo the heavier it will be. Wet wood or bamboo produces more smoke (volatile matters), which is ineffective.

The Relationship Between Moisture Content and High Heating Values

There was a positive relationship between moisture content in dried bamboo and the high heating value (r=0.059 and p>0.05). Moisture content in wet bamboo relates negatively with high heating value (r=-0.188 and p>0.05). This means that dried bamboo produces more heat than wet bamboo. The study shows that the high heating value in the bamboo samples decreases with high moisture content. High moisture content reduces the heat in the bamboo and reduces boiler efficiency (Lyngfelt et al. 2001).

The Relationships Between Moisture Content and Ash Content of The Bamboo

There were variations between moisture contents (wet and dry bases of the bamboo) and ash content. There was

a negative correlation between the moisture content of wet bamboo and the ash content (r=-0.064 and p>0.05), whilst the dry bamboo relates positively with ash content (r=0.330 and p>0.05). These imply that more moisture in the bamboo produces less ash content. It is most likely that the minerals in the bamboo are evaporated in the form of smoke and steam.

The Relationships Between Moisture Content and Density of the Bamboo

There was a positive correlation between the moisture content of wet or fresh bamboo and basic density (r=0.145 and p>0.05). This shows that fresh bamboo is heavier than the similar dried type.

The Relationship Between High Heating Value and Hydrogen and Carbon Contents

The correlation between the high heating value and hydrogen was very strong and positive (r=0.755). A high percentage of hydrogen in the bamboo gives high heating values. This points out that higher hydrogen content is desired in bamboos to increase the high heating values. The relationship between high heating value and carbon was significant (r=0.724 and p=0.05). Carbon is one of the main components of biomass, which increases the high heating value. Therefore, high carbon content is needed in biofuels.

The Relationship Between High Heating Value and Ash Content

There was an intermediate negative correlation between the high heating value and the ash content (r=-0.603 and p>0.05). The bamboo sample which has less ash content produces more heat than bamboo with high ash content. There was a negative correlation between carbon content and the ash content (r=-0.872 and p>0.05). The higher the percentage of carbon contents in the fuel, the lower the ash content. In biofuel production lower ash content is required, so that ash content will not produce slag, foul, and also corrode the conversion plant.

CONCLUSION

The moisture content of fresh bamboo increases the weight of the bamboo. High moisture content reduces the heat value in the bamboo and reduces boiler efficiency. Therefore, fresh bamboos produce more smoke (volatile matters), which may be injurious to human health. Carbon, hydrogen, and oxygen are the main components of biomass that increase the high heating value. Therefore, high carbon, hydrogen, oxygen, and lower ash content recorded in the study predict its suitability as a biofuel material. Fuel properties of *Bambusa vulgaris* are comparable to some wood types, lower to denser wood types, but higher than most of the herbaceous energy crops and agricultural residues. This means that *Bambusa vulgaris* may be a good feedstock for the production of bioenergy in terms of heat, charcoal, biogas, bio-power, and transportation fuel.

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