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CONTENTS

INFLUENCE OF TECTONIC FAULTS ON THE CONDITIONS AND PROPERTIES OF SOME COMPONENTS OF A BIOGEOCENOSIS IN A SUBARCTIC AREA

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ABSTRACT

In geoecology, the nature of the effect of tectonic faults on the environment is not well studied. The influence of natural landscape-geochemical factors on the state and properties of some components of biota of different hierarchical levels is studied at the intersection of two tectonic faults (Velsko-Ust'yanskiy tectonic knot (TK)) in the Arkhangelsk region. Two species of shrubby lichens (*Cladonia stellaris* Opiz. and *Usnea subfloridana* Stirt.) and woody plants (spruce – *Picea abies* L. and Scots pine – *Pinus sylvestris* L.) were chosen as test systems. The field studies were carried out at nine test sites (in the centre, on the periphery and some distance from TK – the background reference point) in different types of forest. The ash content of samples of the lichen *Cladonia* growing in the centre of the TK (1.12–1.22%) is double that in the control area (0.56–0.58%), and for the lichen *Usnea*, it is seven times higher (6.82–6.99% at the centre and 0.97–1.09% in the control area). The ash content of tree bark collected at the centre of TK (1.27–1.29%) is double that at the control site (0.56–0.76%). This indicates a significant accumulation of metals in the vegetation in the TK zone. The accumulation of heavy metals, the low water content of plants, the influence of geomagnetic fields and other factors provoke excessive generation of active oxygen radicals and plants have various physiological, biochemical and morpho-biometric means of combating their adverse effects. The synergism of the cooperative protective action of lichen matrix components on oxidative stress is expressed in terms of changes in biochemical parameters. At the centre of the TK, the lichens contains up to 190 $\mu g\ g^{-1}$ of ascorbic acid, whereas in the control area it does not exceed 130 $\mu g\ g^{-1}$. The content of usninic acid in the centre is 1.5–2 times higher for the *Usnea subfloridana* and is 1.5 times higher for *Cladonia stellaris* compared to the level in the control area.

Keywords: biochemical activity; lichens; pine; spruce; tectonic knot

Introduction

Natural geological processes, unlike technogenic processes, function continuously and result in a global migration of matter into the environment, which has a negative effect on biota. Modes and dynamics of interaction of the lithosphere, biosphere, atmosphere and ionosphere play a significant role in the development of the Earth's crust, primarily the tectonic faults. The features of the structure and properties of tectonic zones determine not only deep degassing and increased relaxation of rocks, but also the conditions for the formation of sources of electromagnetic signals and energy exchange between geophysical fields of different natures, including external and internal. Currently, there is a significant amount of data, indicating the presence of a strong relationship between the processes in the Earth's biosphere, heliogeophysical, meteorological and lithospheric factors, primarily for tectonic faults and their intersections, in convective heat flow. In tectonic fault zones, the geochemical, geophysical and geodynamic fields can jointly affect the biota.

The most interesting are the areas above where tectonic faults intersect (TKs). TKs are complex, three-dimensional bodies that extend to great depths. With the increase in the number of intersecting tectonic faults in an area the degree of fragmentation, permeability and depth of the Earth's crust increases and vertical highly permeable areas develop that over long periods of time, result in an interaction between the crust and the mantle, and a constant flow of fluids and gas from deep within the crust (Kutinov et al. 2009; Kutinov and Chistova 2012) and an inflow of mineralised waters, which contributes to the concentration of a number of elements, including heavy metals. TKs, with a complex conduction field, can be sources of induced eddy currents that change the overall picture of the geomagnetic field (a kind of natural dipole).

The above indicates there are a number of environmental factors associated with tectonic nodes, which can have a joint effect on biological systems at different hierarchical levels. It is likely that such patterns are characterised by special patterns of accumulation, migration and physical and chemical transformation of pollutants (Caine et al. 1996; Evans et al. 1997; Caine and Forster 1999; Rawling et al. 2001; Bense et al. 2013).

Early studies show that the structure of vegetation growing over TKs in the subarctic is different. In these areas the soil is also polluted and there are high levels of heavy metals in the bark of trees, ionisation effects in the atmosphere and differences in the trees, snow cover, clouds and rainfall in summer (Gofarov et al. 2006; Kutinov et al. 2009; Kutinov and Chistova 2012). Thus, in these areas plants are exposed to many physical and chemical effects, which may act synergistically on the plants.

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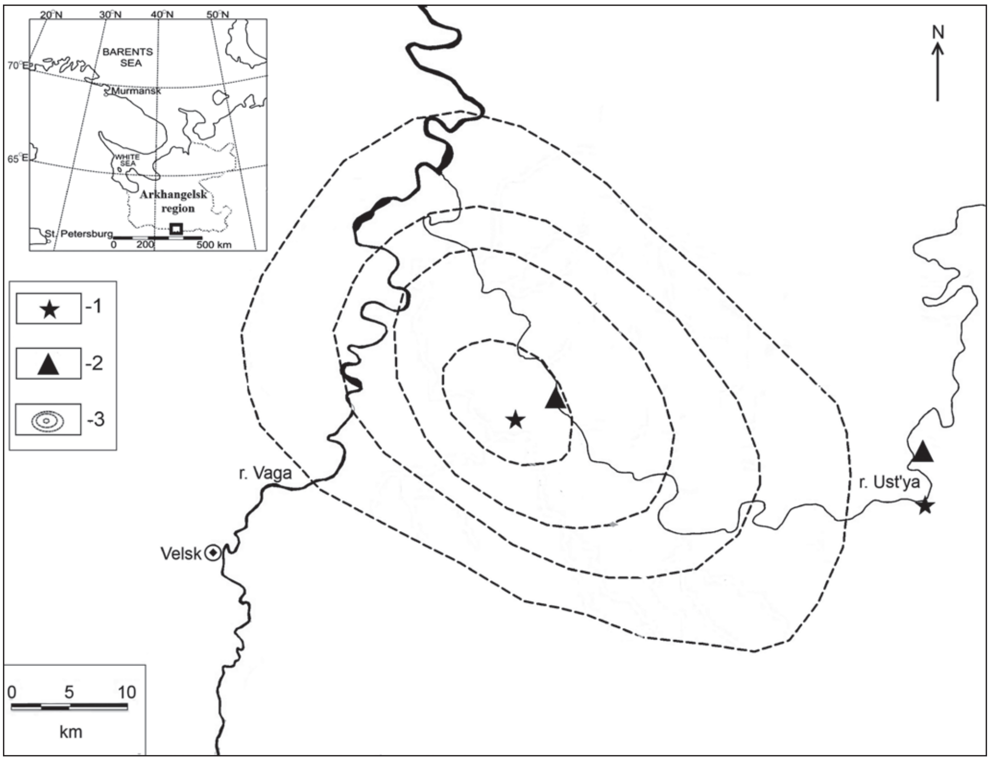

Fig. 1 Map showing the location of the sample plots (SP): 1 – in pine forests; 2 – in spruce forests; 3 – isolines of tectonic dislocation density. 1, 3, 5, 7 – SP in the center of KF; 9 – SP at the periphery of KF; 2, 4, 6, 8 – SP outside the TK zone (control).

The primary reaction of a plant cell to a negative effect is oxidative stress (Beschel 1957; Rydzak 1968; Magomedova 1996; Markovskaya 2010; Brovko et al. 2016). The most important response of plants to stress is a physiological and biochemical restructuring of their metabolic processes. It is known that there are changes in the activity of components of the antioxidant system (AOS) in response to adverse environmental factors such as drought (Zhang and Kirkham 1994), salinity (Meloni et al. 2003), low temperature (Aroca et al. 2001) and increasing concentrations of pollutants in the environment (Chapin 1991; Alscher et al. 1997; Prasad et al. 1999; Chirkova 2002; Polesskaya et al. 2004), etc. However, there are practically no studies on changes in AOS during the ontogenetic development of lichens growing when geological structures are violated (in the region of TKs?).

The goal of this study is to determine how woody plants and lichens have adapted to living in the region of a TK in subarctic conditions in North-West Russia.

Material and Methods

Materials

The plants used in this study were two species of shrubby lichens (*Cladina stellaris* Opiz. and *Usnea subfloridana* Stirt.) and two conifers (Spruce – *Picea abies* L., pine – *Pinus sylvestris* L.). Lichens were identified following Golubkova et al. (1978). The sample plots (SPs) were at permanent test sites established in 2014–2017 in the middle taiga subzone in the district Velsko-Ust'yanskiy TK in the Arkhangelsk region (Fig. 1).

The underlying rocks at the centre of the TK are sandstones, sands and siltstones of the middle Perm, and outside of the TK, variegated clays, sands and marls of the same age. The covering deposits are upper quaternary sands of lake-glacial and alluvial origin. The soils in the centre of the TK are podzolic and illuvial-ferruginous on quartz sands, outside the TK, they are podsolic on a loamy noncarbonate moraine (Atlas of the Arkhangelsk region 1976).

In total, there were nine test areas (Fig. 1, Table 1): four in the centre of the Velsko-Ust'yanskiy TK (SP-C) and four outside (SP-CL), two in a cowberry pine forest, a lichen pine forest, a bilberry, pine and spruce forest in the centre of the TK and in the control area and SP 9 – on the periphery. SPs were selected to be as similar as possible in terms of forest characteristics. The characteristics of forest stands are presented in Table 1.

Table 1 shows that the trees in the SPs have very similar characteristics and differ only in their location relative to the TK (centre and outside the node – control).

In addition, in order to determine the age of the trees 30 cores samples were collected using a core sampler from trees at a height of 1.3 m at each sample plot located in the centre of the TK and in the control area. The age of the forest and any damage to the wood was determined using Lintab 6 and TSAP-Win (version 4.80) (Rinn 2007).

Table 1 Key characteristics: values (means) of the sampled forests.

Sample plot		Height (m)	Normality	Stand composition	Age (yr)	Timber volume, (m^3 ha^{-1})
Bilberry pine forest	SP1-C	17	0.8	7P:3F:B	65	150
	SP2-CL	19	0.7	8P:2F	80	180
Lichen pine forest	SP3-C	12	0.6	10P	90	40
	SP4-CL	10	0.4	10P	100	40
Cowberry pine forest	SP5-C	18	0.7	8P:2B	80	220
	SP6-CL	18	0.7	8P:2B	70	210
Bilberry spruce forest	SP7-C	19	0.7	8F:2B:L	70	250
	SP8-CL	18	0.7	7F:1P:2B	80	240

Note: F – fir-tree, L – larch-tree, B – birch-tree, P – pine-tree

Scanning electron microscopy

The morphology of the samples was investigated using a ZEISS Sigma VP SEM scanning electron microscope (accelerating voltage, 10 kV; detector, InLens). A gold-palladium coating (80:20) no more than 5 nm thick was used. It was obtained using a QUORUM Q150T ES magnetron sputterer.

The biochemical parameters

The biochemical parameters of the two species of lichens that dominate the area studied but differ in their distribution were analysed. Of the soil growing (epigeous) and epiphytic lichens, *Cladonia stellaris* and *Usnea subfloridana*, respectively, were studied. The material was collected in the vegetative period (spring-autumn) from the three SPs (in the centre, at the periphery, outside the TK, which acted as the control) in pine-bilberry forests of similar composition. Two SPs measuring 30 × 30 m were established in each TK zone (centre, periphery) at a distance of 200 m from each other; at the centre (SP5-C) and the periphery of the TK (15 km from the centre – SP9), and outside the TK (50 km from the centre, the control – SP6-C) (Fig. 1). Five to ten samples of both species of lichen were collected from each SP. A total of 160 samples of lichen were analysed. The composition of the vegetation in pine-bilberry forests is presented in Table 2.

Analysis

The water content in the samples was determined gravimetrically, the content of mineral substances was determined gravimetrically after dry ashing (500 °C). Elemental analysis of the lichens was done using an Euro EA-3000 element analyser (configuration [CNHS]) (EuroVector, Italy). The content of heavy metals and biogenic elements was determined using a XRF-1800 series waveguide X-ray fluorescence spectrometer (Shimadzu, Japan).

Estimation of acids

The content of ascorbic acid (AA) in lichens was determined spectrally using a UV-1800 spectrophotometer (Shimadzu, Japan) and 2.6-dichlorophenolindophenol (Tilmans paint) according to (Voskresenskaya 2006). Optical density was measured at 515 nm. The change in the staining intensity of the control and test sample is proportional to the amount of AA in the plant extract.

Usnic acid (UA) was extracted using acetone and quantified using a LCMS-2020 liquid quadrupole LCM-mass spectrometer (Shimadzu, Japan) according to (Brovko et al. 2017). Chromatography conditions were: mobile phase – 0.5% aqueous solution of formic acid and acetonitrile in a volume ratio of 30:70; column Restek Ultra C18 100 mm (length) × 3 mm (diameter), fixed phase grain size 3 µm, mobile phase flow rate 0.5 ml min^{-1}, sample introduction volume 5 µl. Using a standard Sigma-Aldrich UA sample, calibration curves of the peak area versus the concentration in the range from 1 µg l^{-1} to 0.1 mg l^{-1} were constructed. The calibration curves

Table 2 Composition of the vegetation in pine-bilberry forests in the district Velsky-Ust'yanskiy TK in the Arkhangelsk Region.

Location of the trial plot in relation to KF		
SP 6-C (control)	**SP 9**	**SP 5-C (center)**
Pine (*Pinus sylvestris* L.)	Pine (*Pinus sylvestris* L.)	Pine (*Pinus sylvestris* L.)
Spruce (*Picea abies* L.)	Spruce (*Picea abies* L.)	Spruce (*Picea abies* L.)
Birch (*Betula pendula* Roth)	Birch (*Betula pendula* Roth)	Birch (*Betula pendula* Roth)
Cowberry (*Vaccinium vitis-idaea* L.)	Cowberry (*Vaccinium vitis-idaea* L.)	Cowberry (*Vaccinium vitis-idaea* L.)
Blueberry (*Vaccinium myrtillus* L.)	Blueberry (*Vaccinium myrtillus* L.)	Blueberry (*Vaccinium myrtillus* L.)
Green mosses	Green mosses	Green mosses
Lichens	Lichens	Lichens
	Heather (*Calluna vulgaris* L.)	Juniper (*Juniperus communis* L.)
		Melampyrum (*Melampyrum pratense* L.)

are linear over the concentration range recorded in this study with a correlation coefficient of 0.99.

Statistical analysis

All analytical measurements were performed in triplicate. The results of the experiments are presented in the form of an average of the arithmetic means and their standard error. To establish the statistical relationships between the parameters, Student's t-test was used at a confidence level of P = 95%. A statistical analysis was performed in Python (version 2.7.12, 2016) in the SkiPy package (version 0.18.1, 2016) using the Statistica 10 software package ("StatSoft", USA).

Results and Discussion

Vegetation is a good indicator of changes in environmental conditions due to terrain features. Lichens (*Cladonia*, *Usnea*) are widespread throughout the Arkhangelsk region, growing in both dry sandy and very wet conditions. Due to their long life, they are continuously subjected to stress over a long period of time and as a consequence have evolved protective measures. Therefore, these species are convenient models for studying the various modes of adaptation of vegetation to growing on these tectonic structures.

The reactions of the multilevel structural and functional organisation of plants to the influence of tectonics is mixed. The accumulation of heavy metals, the lack of moisture and the influence of geomagnetic fields provoke changes in the biochemical cycles of plant development, which are manifested both at the macro and micro structural levels. Visually there is an obvious retardation of growth, excessive branching and other morphological abnormalities, which may indicate irreversible changes in the plants due to a restructuring of their metabolism.

In tectonic fault zones there are multi-stemmed trees, the causes of which have not been fully elucidated. The indirect evidence of these abnormalities is that they were induced by the geological heterogeneities in the Earth's crust. The high frequency of "witches broom" in these zones is not due to infections but to mutations (Korovin et al. 2003). In forests in the Trans-Urals in areas with tectonic faults, birch trees are highly polymorphic (Mamaev 1973; Makhnev 1987). An example of this is Karelian birch, which results from abnormal cambial activity

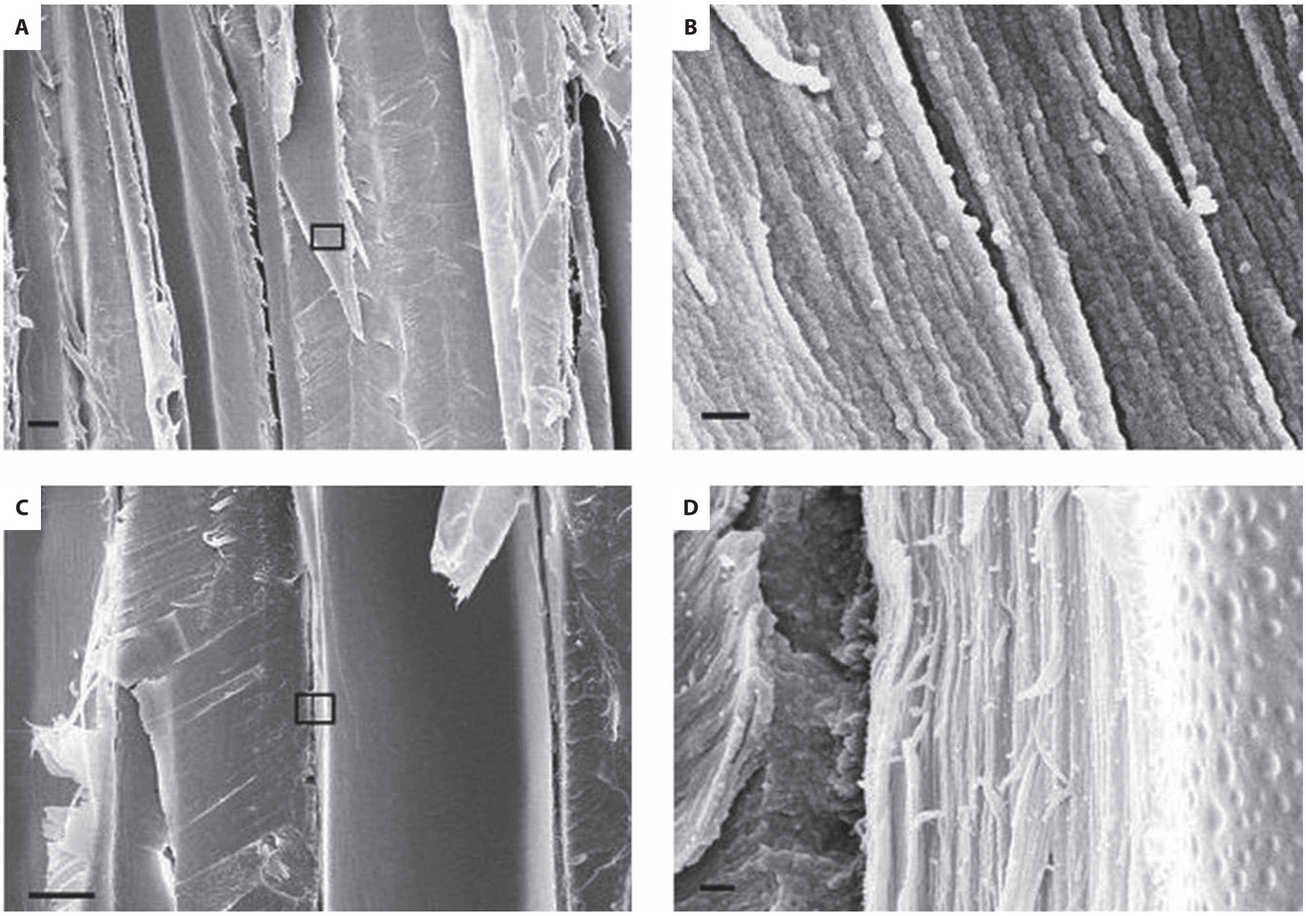

Fig. 2 Micrographs of chips from core samples; (a) cleavage of the cell wall showing the typical orientation of microfibrils in the S_2 layer (control); (b) the area selected in Fig. 2a highly magnified; (c) cleavage of the cell wall with a fibre orientated parallel to the fibre in the microfibril in the S_2 layer (centre of the node); (d) the area selected in Fig. 2c highly magnified. Bars: (a), (c) 10 microns; (b) 100 nm; (d) 200 nm.

and a reduction in the distribution of photosynthates by the phloem to the xylem and increase in the distribution to the cortex. Structural and functional deviations from normal growth and development in plants may indicate an increase in cell growth and differentiation, phenol metabolism and respiration when under stress.

In transverse chips from the core samples, there is sometimes an atypical orientation of the cellulose relative to the fibre axis in microfibers in the S_2 layer of the cell wall (Fig. 2).

The angle can be up to 0° (this conclusion is not statistically significant).

Increasing the thickness of the cell wall reduces its permeability to toxic metabolic products and heavy metals, and protects the cellular components of the plants. Woody plants actively adapt to unfavourable environmental conditions by growing more slowly, as is clearly shown by the width of their annual rings, which are narrower in the centre of the TK than in the control area.

The soil in the different types of forest in the TK zone contains significantly more of the typical lithogenous macro- and microelements (mg g^{-1}): Si (4.8–36.5), Ca (2.7–4.0), Al (3.4–4.9), Fe (2.2–4.3), Na (0.9–1.3), Mg (0.4–1.1), Mn (0.23–0.31), Ti (0.1–0.6), Cu (0.05–0.06) and Zn (0.04–0.07) than in the control areas: Si (1.8–26.1), Al (0.8–1.83), Ca (0.7–1.57) and Na (0.7–1.0).

It is known that the elemental composition of plants is relatively stable. However, the geochemical environment and ecological conditions in the TK zone can result in lichens accumulating significantly more of certain elements. The mineral composition of the lichens is correlated with the mineral composition of the soil covering the TK. The content of macro- and microelements in the ash of lichens growing there is greater than in the control area, which indicates that their mineral composition reflects the geochemical features of the landscape. Lichens growing at the periphery and in the centre of the TK contained a number of typical lithogenic elements: Si > Al > Ca > K > Mg > Fe > Mn > Cu > Zn. The ash content of the lichen *Cladonia stellaris* growing in the centre of the TK is double (1.12–1.22%) that of the control (0.56–0.58%), and for the lichen *Usnea subfloridana*, it is 7 times higher in the centre of the TK (6.82–6.99%) than in the control area (0.97–1.09%). That is, lichens growing tectonic fault zones accumulate significant amounts of various metals. The ash content of the bark of tress growing in the centre of the TK (1.27–1.29%) is double that of the control (0.56–0.76%), which again reflects the lithochemical features of the sedimentary rocks in the upper part of the TK. The chemical elements in the soil are possibly taken up by the roots of the trees in which it accumulates in the wood and bark, and in lichens. In addition, the lower average monthly rainfall in summer at the centre of the TK affects the increase in the content of mobile forms of metals in the upper horizon of the soil (0–20 cm), which creates extremely unfavourable conditions for plant growth.

Lichens are an integral component of most plant communities. Their diversity and distribution are largely determined by the ecological conditions in a region. The high sensitivity of lichens to stress, and, accordingly, their high rate of biochemical reactions, are due to the fact that the metabolic equilibrium between the photobiont and the mycobiont is easily disturbed, as evidenced by the indices of biochemical reactions taking place in lichens (the content of ascorbic acid, phenolic compounds, lichen acids and so on) (Brovko et al. 2015; Brovko et al. 2016).

It is known that ascorbic acid (AA), due to its ability to easily donate electrons, is the leading non-enzymatic antioxidant (Chapin 1991). In the centre and at the periphery of the TK, the content of AA in lichens is higher (Fig. 3) than in the control area.

This activation of free radical oxidation is a nonspecific form of resistance shown by lichens when subjected to various stressors (Chapin 1991).

Phenolic compounds (PCs) is one of the most common classes of secondary metabolites, the formation of which is characteristic of virtually all plant cells. They take part in a wide variety of physiological processes, such as photosynthesis, respiration, formation of cell walls and resistance of plants to the action of heavy metals (Brovko et al. 2017). In the literature, there are more than 1000 PCs characteristic of lichens, the so-called "li-

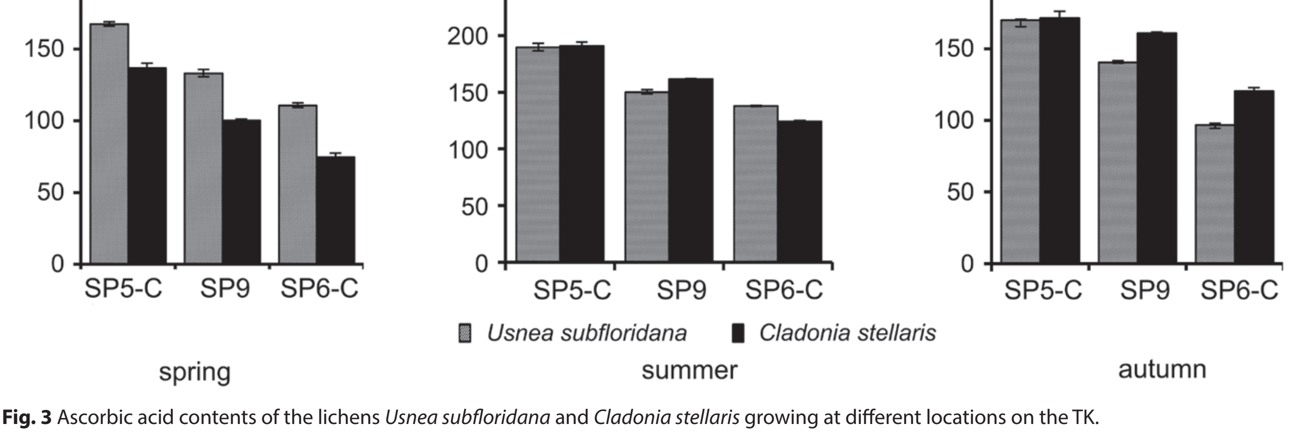

Fig. 3 Ascorbic acid contents of the lichens *Usnea subfloridana* and *Cladonia stellaris* growing at different locations on the TK.

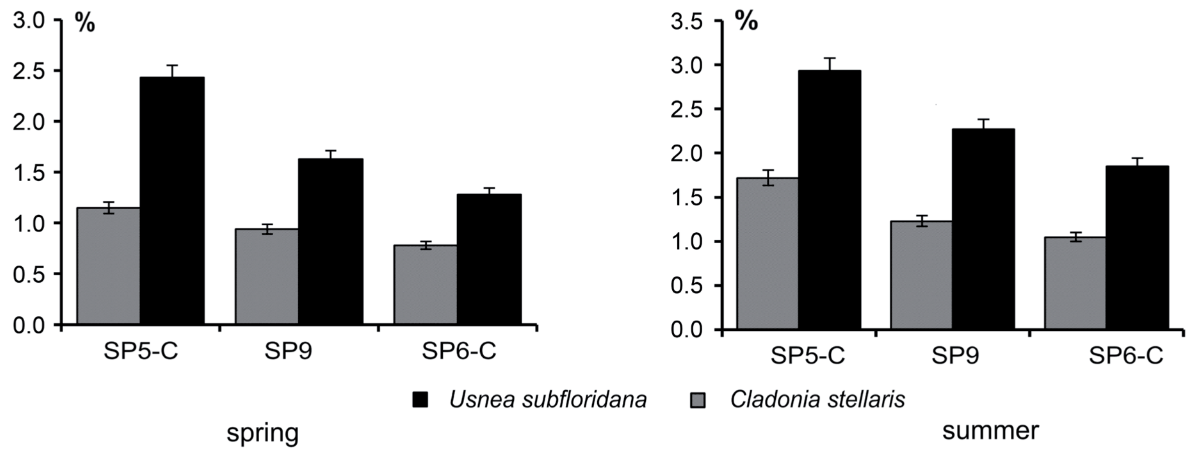

Fig. 4 Usnic acid content of lichens, % of *a.d.w.*

chen substances" (Molnar and Farkas 2010), of which the most commonly found is tallow and the well-known usnic acid (UA) (Brovko et al. 2017).

The changes in the content of acids in lichens growing in different environments is of particular interest. Our studies show that the content of UA, a typical secondary metabolite of lichens (Brovko et al. 2017), in *Usnea subfloridana* and *Cladonia stellaris* varied depending where they grew in the TK zone (Fig. 4).

There are seasonal fluctuations in the amount of UA, which indicate that lichen acids actively participate in metabolism and sometimes accumulate in the thallus in significant amounts. An important function of these acids is regulatory. An increase in their content promotes photosynthesis of algal cells – lichen symbionts, which dramatically increases the amount of organic matter needed for the symbiotic fungus. The increase in UA at the centre of the TK by 1.5–2 times in the lichen *Usnea subfloridana* and 1.5 times in the lichen *Cladonia stellaris* compared to the control, indicates the high adaptive capacity of lichen symbionts to combat the adverse environmental factors that these lichens are exposed to.

An important role of lichen acids is the allelopathic interaction with other components of phytocenoses, in particular, tree-destroying fungi. Analysis of samples of wood to determine the number of trees infected with the root fungus (*Heterobasidion annosum* Fr.) in the centre of the TK revealed that less than half of the spruce and none of the pines were infected compared with the control. Apparently, lichens growing on trees make them more resistant to wood-destroying fungi (Henningsson and Lundstrom 1970), which is due to the high fungicidal effect of lichen acids.

Conclusion

The results of this research show that the adverse conditions at TK zones induce in plants biochemical and biophysical reactions that enable them to survive in these areas. At these sites, the structure of the cell walls of woody plants differ in a way that reduces their permeability to toxic metabolic products and heavy metals. The growth of some of the trees at the centre of the TK was abnormal because the conditions experienced there adversely affected the activity of their cambium. The synergism between trees and lichens provides ascorbic and lichen acids that inhibit the oxidation of free radicals. Thus, there are two ways of adapting to environmental stress. One is associated with a change in the structure of the cell walls of plants (barrier) and the other with the activation of systems that detoxify reactive oxygen species and free organic radicals.

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REFERENCES

Alscher RG, Donahue JL, Cramer CL (1997) Reactive Oxygen Species and Antioxidant: Relationships in Green Cells. Plant Physiol 100: 224–233, https://doi.org/10.1111/j.1399-3054.199 .tb04778.x.

Aroca R, Irigoyen JJ, Sanchez-Diaz M (2001) Photosynthetic characteristics and protective mechanisms against oxidative stress during chilling and subsequent recovery in two maize varieties differing in chilling sensitivity. Plant Sci 161: 719–726, https://doi.org/10.1016/S0168-9452 (01)00460-5.

Atlas of the Arkhangelsk region (1976) General administration of geodesy and cartography, Moscow, USSR.

Bense VF, Gleeson T, Loveless SE, Bour O, Scibek J (2013) Fault zone hydrogeology. Earth-Scie Rev 127: 171–192, https://doi.org/10.1016/j.earscirev.2013.09.008.

Beschel RE (1957) A project to use Lichens as indicators of climate and time. Arctic Alpine Res 10: 200–215.

Brovko OS, Palamarchuk IA, Boitsova TA, Bogolitsyn KG, Valchuk NA, Ivakhnov AD (2015) Comparative analysis of traditional and modern extraction methods of usnic acid from raw lichen material. Fund Res 11: 659–663, https://doi.org/10.17513/fr.39481.

Brovko OS, Palamarchuk IA, Sloboda AA, Boytsova TA, Gagushkina AA, Valchuk NA, (2016) The influence of stress factors on the chemical composition of lichens genus Cladonia of Euro-Arctic region. Adv Nat Sci: 20–24, https://doi.org/10.17513/use.36072.

Brovko OS, Ivakhnov AD, Palamarchuk IA, Boitsova TA (2017) Supercritical Fluid Extraction of Usnic Acid from Lichen of *Cladonia* Genus. Russ J Phys Chem B+ 11: 1306–1311.

Caine JS, Evans JP, Forster CB (1996) Fault zone architecture and permeability structure. Geology 24: 1025–1028, https://doi.org/10.1130/0091-7613(1996)024<1025:FZAAPS>2.3.CO;2.

Caine JS, Forster CB (1999) Fault zone architecture and fluid flow: Insights from field data and numerical modeling. In: Haneberg WC, Mozley PS, Moore JC, Goodwin LB (eds) Faults and subsurface fluid flow in the shallow crust. American Geophysical Union, Washington DC, USA, pp. 101–127.

Chapin FS (1991) Integrated responses of plants to stress. A centralized system of physiological responses. BioScience 41: 29–37, https://doi.org/10.2307/1311538.

Chirkova TV (2002) Physiological basis of plant tolerance. Izdatelstvo Sankt-Peterburgskogo Universiteta, Saint Petersburg, Russian Federation.

Evans JP, Forster CB, Goddard JV (1997) Permeability of fault-related rocks, and implications for hydraulic structure of fault zones. J Struct Geol 19: 1393–1404, https://doi.org/10.1016/S0191-8141(97)00057-6.

Gofarov MYu, Kutinov YuG, Bolotov IN (2006) Landscapes of the Belomorsko-Kuloyskoe plateau: tectonics, bedrock, topography and vegetation. Ural Branch of RAS, Ekaterinburg, Russian Federation.

Golubkova NS, Savich VP, Trass HH (1978) The determinant of lichens of the USSR. Vol. 5. Cladoniaceae-Acarosporaceae. Nauka, Leningrad, USSR.

Henningsson B, Lundstrom H (1970) The influence of lichens, lichen extracts and usnic acid on wood destroying fungi. Mater Organismen 5: 19–31.

Korovin VV, Novitskaya LL, Kurnosov GA (2003) Structural anomalies of the stem of woody plants. Publishing House of the Moscow State University of Forest, Moscow, Russian Federation.

Kutinov YuG, Chistova ZB, Belyaev VV, Burlakov PS (2009) The Influence of tectonic disturbances (degassing, induced currents, variations) of the North Russian plate on the environment (on the example of the Arkhangelsk region) Vestnik KRAUNZ Earth Sci 2: 77–89.

Kutinov YuG, Chistova ZB (2012) Complex model of geospheres interaction processes in the tectonic nodes at the North Russian plate. Electronic scientific publication Almanac Space and Time 1: 9 [online 22 June 2018], http://www.j-spacetime.com/actual%20content/t1v1/1109.php.

Magomedova MA (1996) Lichens as a component of northern ecosystems and the monitoring object. Problems of ecological monitoring and ecosystem modelling. XVI. Gidrometeoizdat, Sankt-Petersburg Russian Federation.

Makhnev AK (1987) Intraspecies variation and population structure of birches of sections Albae and Nanae. Nauka, Moscow, USSR.

Mamaev SA (1973) Forms of intraspecific variation of woody plants. Nauka, Moscow, USSR.

Markovskaya EF, Sergienko LA, Shklyarevich GA, Sonina AV, Starodubtseva AA, Smol'kova OV (2010) Natural complex of the White Sea coasts. KarRC of RAS Publ., Petrozavodsk, Russian Federation.

Meloni DA, Oliva MA, Martinez CA, Cambraia J (2003) Photosynthesis and Activity of Superoxide Dismutase, Peroxidase and Glutathione Reductase in Cotton under Salt Stress. Environ Exp Bot 49: 69–76, https://doi.org/10.1016/S0098-8472(02)00058-8.

Molnar K, Farkas E (2010) Current results on biological activities of lichen secondary metabolites: a review. Z Naturforsch 65: 157–173.

Polesskaya OG, Kashirina EI, Alekhina ND (2004) Changes in the activity of antioxidant enzymes in leaves and roots of wheat depending on the form and dose of nitrogen in the medium. Plant Physiol 51: 686–691.

Prasad KVSK, Saradhi PP, Sharmila P (1999) Concerted Action of Antioxidant Enzymes and Curtailed Growth under Zinc Toxicity in Brassica juncea. Environ Exp Bot 42: 1–10, https://doi.org/10.1016/S0098-8472(99)00013-1.

Rawling GC, Goodwin LB, Wilson JL (2001) Internal architecture, permeability structure, and hydrologic significance of contrasting fault-zone types. Geology 29: 43–46.

Rinn F (2007) TSAP – Win TM Professional. Zeitreihenanalyse und Präsentationfür Dendrochronologie und verwandte Anwendungen. Benutzerhandbuch RINNTECH, Heidelberg.

Rydzak J (1968) Lichens as indicators of the ecological conditions of the habitat. Annales Universitatis Mariae Curie-Sklodowska 23: 131–164.

Voskresenskaya OL (2006) Great Workshop on bioecology. Part 1. Textbook. Mari state University, Yoshkar-Ola, Russian Federation.

Zhang J, Kirkham MB (1994) Drought-Stress Induced Changes in Activities of Superoxide Dismutase, Catalase, and Peroxidase in Wheat Species. Plant Cell Physiol 35: 785–791, https://doi.org/10.1093/oxfordjournals.pcp.a078658.

A QUANTITATIVE APPROACH TO LAND USE PLANNING USING GIS – A CASE STUDY OF CHABAHAR COUNTY, IRAN

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ABSTRACT

Land use planning aims to formulate activities, administer potential changes and prevent incompatible changes. The aim of this study is to prepare a land use plan for Chabahar County, Iran, based on a quantitative model using GIS. This study involves two main stages. First, the overlaying of geographical maps and preparing ecological capability maps of different land uses, like forestry, agriculture, range management, environmental conservation, ecotourism and development of villages, urban areas and industry using GIS. The second stage involves prioritizing the land uses taking into consideration the ecological and socio-economic characteristics of the study area and using a quantitative model. The results indicate that the proposed model provides better land use planning than Iran's Makhdoom model. The new model provides clearer and more suitable uses for the land than those used currently. The results also indicated that the maximum area of proposed uses (52.17%) was related to ecotourism, showing this land use had high potential and socio-economic demands in study area. Also, minimum area of proposed uses was related to development.

Keywords: Chabahar County; GIS; land use optimatization; land use planning, modified Makhdoom's model

Introduction

Land use planning is useful for planning the development of an area as it aims to formulate activities, administer potential changes and prevent incompatible changes, and so ensure sustainability (FAO 1993; Van Lier 1998; Makhdoom 2001; Cools et al. 2003; Jozi 2010).

In addition to increasing food production, the loss of valuable land by degradation and deterioration should be restricted as much as possible. Due to potentially rapid degradation rates and slow regeneration, land is a limited non-renewable natural resource. Degradation results in a loss of production and a reduction in the capabilities of land to perform its functions (Ward et al. 1998; NEMA 2004; Abu Hammad and Tumeizi 2010; Barzani and Khairulmaini 2013; Jafari and Bakhshandehmehr 2013).

Although perfect land use planning is a complex decision making process, modern GIS technologies have made this task easier in two ways: (i) They allow one to work simultaneously on a large number of datasets, (ii) Some of the methods, techniques or models can be embedded in GIS used for the suitability analysis of areas of land (Pauleit and Duhme 2000; Swanson 2003; Nouri and Sharifipour 2004; Gad 2015; Atalay 2016). For a more accurate land use planning a wider range of social, economic, physical and environmental indicators need to be included. Inclusion of geographical data in GIS allows these indicators to be used in a more sophisticated way in the decision making process of land use planning. However, for handling the datasets in a GIS environment it is necessary to include a geographical database management system – especially, when the datasets are robust and complex. To build such a geographical database it is essential, first to prepare a conceptual model so that the data requirements and their interrelations are well defined and that the database can be used to store, modify and query the security of the data. Then, a number of Multi-Criteria Decision Making (MCDM) models or techniques embedded in the GIS can be used for land use suitability analysis, where the importance of each indicator of land use is determined in a more sophisticated way based on subjective and or objective judgments. The literature indicates that Boolean and AHP methods, which are kinds of MCDM techniques, can be used for land use planning within a GIS (Bojo'rquez-Tapia et al. 2001; Biswas and Baran Pal 2005; Peel and Lloyd 2007; Gandasasmita and Sakamoto 2007; Oyinloye and Kufoniyi 2013; Farashi et al. 2016; Allaouia et al. 2018).

Taking the above into consideration, the aim of this research is to prepare a quantitative method for the land use planning of the area studied within a GIS.

Materials and Methods

Chabahar County covers an area of 24,729 km^2 and is located in the Sistan and Baluchistan province in the southeastern part of Iran (Fig. 1). Chabahar city is located between longitude 60°37´E and latitude 25°17´N. This county is located near the warm waters of the Oman Sea and has a humid and warm climate. A systematic method known as the Makhdoom Model (Makhdoom 2001) was used for the analysis of maps in relation to the ecological and socio-economic resources of the area studied. This model is based on an applied and simple Boolean (binary) model.

Several maps were used to evaluate the ecological resources of the area studied, the Digital Elevation Model (DEM), slope and aspect, soil data, erosion, geology, iso-precipitation (iso-hyetal), iso-thermal, iso-evapora-

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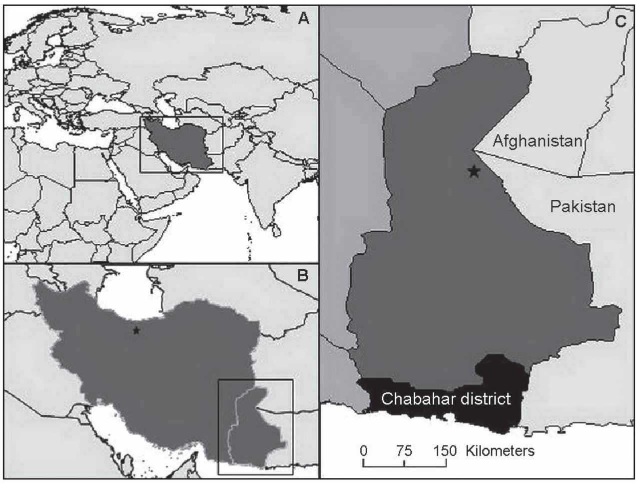

Fig. 1 Map showing the location of Chabahar Province in Iran.

tion, climate, canopy percentage and type and water resources data. These data were gathered from the records of different departments of the Ministries of Agriculture and Energy and Meteorology in Iran. The data obtained consists of two types: 1) attributed data and 2) GIS maps.

Fig. 2 shows the conceptual framework of land use planning for the proposed model. In fact, different ecological capability models based on the Makhdoom method were used to evaluate ecological capability of different land uses including forestry, agriculture, range management, environmental conservation, ecotourism and development of villages, urban areas and industry (Makhdoom 2001). Based on these models, ecological suitability for forestry, agriculture, range management, environmental conservation, ecotourism and development of villages, urbans and industry were 7, 7, 4, 3, 3 and 3, respectively. The best and worst suitability's are the first and the last in each model, respectively. Note that the ecological suitability assessment was based on Boolean algebra. The good and moderate ranges are shown in Table 1.

Based on Fig. 2 and ecological suitability maps, a land use planning map was prepared, which was done by intersecting capability maps. Then the process of land use planning was done by evaluating four scenarios including: a) present land utilization in the area studied and b) the economic, c) social and d) ecological needs of this area. All land uses were ranked for each scenario and then scored from 10 to lower base on their ranks and ecological capability. For example if in one scenario, rank of forestry was placed in the third rank and its ecological capability was class two in a land unit; its score in first step was given 8 and then one score is lowered for its capability reduction (class two) that makes its score number 7 for forestry in the land unit. This means that this one point reduction for forestry in three other scenarios was repeated because of one place of reduction compared to first class of ecological capability. If ecological capability class was class three, the reduction in each scenario would be two.

Ranking in the first scenario was done on the basis of current land use. For other scenarios a questionnaire was completed by 81 experts who ranked the different land uses for each scenario based on their knowledge and experience of the area studied. Averages of the results were used to rank different land uses in each scenario. Questionnaire filling is a good method for determining the socio-economic needs of an area, which depend on many things: socio-political characteristics, population composition, relative earnings, immigration, present land utilization, agriculture and animal husbandry, hygiene, health, education and other public services.

To achieve a systematic analytical model, all maps were layered using a vector format in an ArcGIS software environment. These maps were operated using ArcGIS and the appropriate utilization of each land unit was determined and prioritized. The appropriate utilizations are those that have the highest scores in the different scenarios.

Some of the processes were modified such as preparation of the environmental units and using the current land use map. In this research, the current systemic analysis for preparing environmental units was not utilized for assessing the ecological capability maps and land use planning by the quantitative model. It may be used only

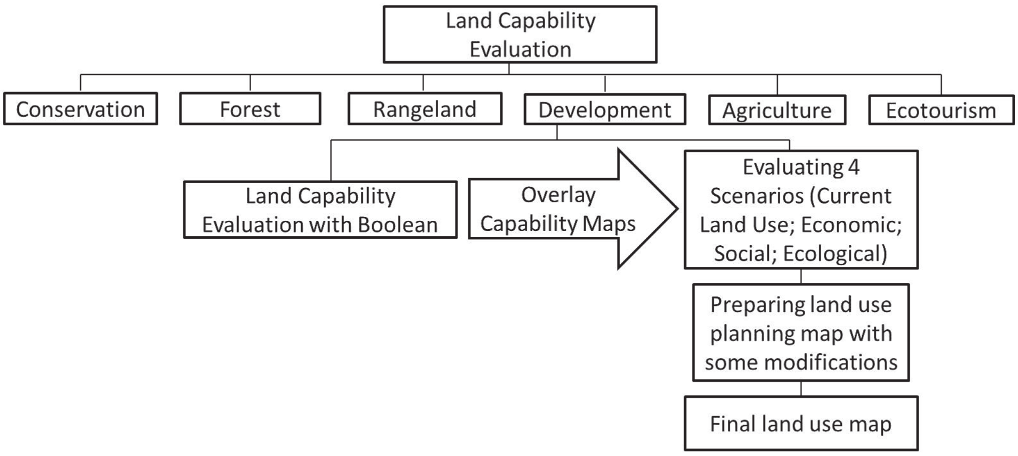

Fig. 2 The conceptual framework of the proposed model for land use planning.

Table 1 Moderate and Good classes for every use.

Indicators	Class	Forestry (class 1–4)	Agriculture and range management (class 1–4)	Ecotourism (intensive) (class 1–2)	Development (class 1–2)
Altitude (m)	Good	0–1000	–	–	400–1200
	Good to moderate	0–1000			0–400, 1200–1800
	moderate	0–1400			–
	Mostly moderate	400–1800			–
Slope (%)	Good	0–25	0–5	0–5	0–12
	Good to moderate	0–35	5–8	5–15	12–20
	Moderate	0–45	–	–	–
	Mostly moderate	0–55	8–15	–	–
Precipitation (mm)	Good	>800	Warm and moderate (Mediterranean to humid)	–	501–800
	Good to moderate	>800	Warm, moderate and cold (semi–arid to humid)		51–500, >800
	Moderate	>500	Warm, moderate, cold and super cold (arid to humid)		–
	Mostly moderate	>500	–		–
Temperature (°C)	Good	18–21	–	21–24*	18.1–24
	Good to moderate	18–21		18–21, 24–30	24.1–30, <18
	Moderate	<18, 18–30		–	–
	Mostly moderate	<18, 18–30		–	–
Sunny days*	Good to moderate	–	–	>15	–
	Moderate			7–15	
Relative humidity (%)	Good to moderate	–	–	–	40.1–70
	Moderate				<40, 70–80
Soil Texture & Type	Good	Brown soil and forest semi humid to loam clay texture	Clay, loamy clay, humus	Usually moderate	Moderate (often)
	Good to moderate	Brown soil and forest semi humid to loam clay texture	Clay, loamy clay, humus clay, sandy loamy clay, sandy clay loam, clay loam, loam	Coarse, light, heavy	Light (often)
	Moderate	Brown soil to clay with loamy texture	Clay loam, loamy sand, loam clay sand, clay loam sandy, sand	–	–
	Mostly moderate	Brown rendezina to clay with loamy texture, regosols brown soil, litosols to sand with loamy texture	Clay, loam clay, clay loam, loam	–	–
Drainage	Good	Moderate to perfect	Perfect	Good	Good
	Good to moderate	Moderate to good	Good	Moderate to poor	Moderate
	Moderate	Rather incomplete to good	Moderate to incomplete	–	–
	Mostly moderate	Rather incomplete to moderate	–	–	–
Depth	Good	Deep	Deep	Deep	Deep
	Good to moderate	Deep	Moderate to good	Semi deep	Semi deep
	Moderate	Moderate to good	Low to moderate	–	–
	Mostly moderate	Moderate to good	–	–	–
Structure	Good	Granulations fine to moderate with a bit of gravel	Granulations fine to moderate with no gravel, with little erosion	Perfect evolution	Slight erosion with granulation moderate
	Good to moderate	Granulations fine to moderate with gravel	Granulating fine to moderate, no gravel, low to moderate erosion	Moderate erosion	Granulations fine, coarse and moderate with moderate erosion

Structure	Moderate	Granulations fine to moderate with gravel	Granulations moderate to coarse with gravel, moderate erosion	Moderate erosion	Granulations fine, coarse and moderate with moderate erosion
	Mostly moderate	Granulations fine to moderate with rubble, low to moderate erosion	–	–	–
Fertility	Good	Perfect	Perfect	Good to moderate	Good
	Good to moderate	Good	Good	Low	Moderate
	Moderate	Moderate to good	Moderate	–	–
	Mostly moderate	Low to moderate	–	–	–
Canopy Cover (%)	Good	>80	–	Forest lands (with canopy cover > 50%)	0–25
	Good to moderate	60–80	–	Forest lands (with canopy cover 5–50%)	26–50
	Moderate	50–70	–	–	–
	Mostly moderate	40–60	–	–	–
Annual Growth (m^3)	Good	> 6	–	–	–
	Good to moderate	To 6			
	Moderate	To 5			
	Mostly moderate	To 4			
Quantity of water for everyone (l/day)	Good	–	6000–10000**	> 40	< 225
	Good to moderate	4000–6000	12–39.9	150–225	
	Moderate	3000–5000	–	–	
	Mostly moderate	To 3000	–	–	
Lithology	Good	Limestone and dolomite, shale, clay stone, Conglomerate and marl type 1	–	Pyroclastic rocks, granite ophiolite of a mixture of colours, sand dunes, continental shelf sediments	Sandstone, Ophiolite of a mixture of colours, continental shelf sediments
	Good to moderate	Limestone and dolomite, intermediate pyroclastic rocks of eocene, shale, clay stone, conglomerate and marl type 1, floodplain, ophiolite of a mixture of colours		Limestone and Dolomite, sandstone, loess, schist and gneiss and amphibolite, quartzite, alluvial fans, flood plain	Limestone and dolomite, intermediate pyroclastic rocks of eocene, granite, alluvial fans, shale, clay stone, conglomerate, loess, alluvial terraces
	Moderate	Limestone and dolomite, intermediate pyroclastic rocks of eocene, shale, clay stone, conglomerate and marl type 1, granite, schist and gneiss and amphibolite, floodplain, ophiolite of a mixture of colours		–	–
	Mostly moderate	Limestone and dolomite, intermediate pyroclastic rocks of eocene, sandstone, shale, clay stone, conglomerate and marl type 1, granite, schist and gneiss and amphibolite, floodplain, ophiolite of a mixture of colours, loess		–	–

* in spring & summer seasons; ** m^3/ha

for assessing small areas with a low diversity (e.g. small watershed). Hence, for assessing larger areas (e.g. large watersheds, counties and provinces), preparation of environmental units involves not using the same amount of information used in the ecological capability models. So, in the present study all indicator maps related to different ecological capability models were overlaid in GIS. Other modifications of the processes that were done for assessing the land use planning model included:

a) Prioritization of each use based on the highest score obtained by summing the scenarios' scores (ecological, economic, social, area) (Makhdoom 2001).

b) Because of the socio-economic position of the population, especially in rural areas, the following land uses were included in the land use planning process:
   1) Irrigated land.
   2) Settlement lands (urban, rural and industrial area).
   3) Dense forests taking into consideration compatibility of uses (e.g. conservation).
   4) Lakes and river beds.

Finally, land use planning maps for Chabahar County were developed considering the ecological and socio-economic characteristics of the area. The process of evaluation included the steps presented in Fig. 2.

Results

For each model, the related indicators were overlaid and then the land capability maps were assessed. The capability maps are shown in Figs 3 to 8 and the percentage of the area suitable for different uses is presented in Table 2.

After that, land capability maps were overlaid and land use planning map (Fig. 9) by quantitative approach was assessed. A comparison of the percentage of the land currently in different land use categories and that proposed by the land use maps is presented in Table 3. The main results of this comparison is that the areas currently assigned to forestry and range management are greater than that proposed by the land use model. A lot of barren land in this area could potentially be used for other purposes, such as ecotourism and environmental conservation. Fig. 9 and Table 2 also show a maximum percentage area of 52.17% is suitable for ecotourism. Also, little of area is currently used and suitable for future development.

Table 2 Percentage of area suitable for different uses.

Land Type	Class	Percent
Agriculture	2	0.03
	3	2.72×10^{-5}
	4	0.12
	5	79.90
	6	19.96
	7	4.47×10^{-7}
Range management & dry farming	1	0.14
	2	79.90
	3	19.96
	4	4.47×10^{-7}
Forestry	4	0.89
	5	15.90
	6	11.70
	7	71.48
Conservation	1	35.79
	2	22.30
	3	41.90
Ecotourism	1	2.83
	2	85.82
	3	11.34
Development of urban and rural areas, industry	2	0
	3	100

Table 3 Comparison of the percentage of the land currently under different types of land use and that proposed by the land use maps.

Land Type	Percentage of current land use	Percentage of proposed land use
Forestry	4.80	0.85
Ecotourism	–	52.17
Urban, rural and industrial development	0.03	0.03
Irrigated	0.47	0.22
Range management	48.33	2.66
Dry farming	10.30	0.27
Environmental conservation	35.79	6.50
Ecotourism-conservation	–	35.44
Saline land	–	–
Bare land	35.30	1.05

Discussion and Conclusions

Arid and semi-arid regions in Iran are undergoing rapid desertification in response to climate warming and anthropogenic disturbances. Hence, it should be noted that the establishment of the best land use is needed for land improvement. Thus, there is a need to improve land use planning. Land degradation can be due to natural hazards, direct and indirect causes. Direct causes include unsuitable land use and inappropriate land management practices, for example cultivation of steep slopes (Masoudi 2010; Masoudi et al. 2018). Some anthropogenic activities like deforestation, using rangelands for cultivation, mining and urbanization, destroy the natural vegetation and degrade the land. All these activities have to be controlled by incorporating the capacity of natural vegetation to sustain them (Masoudi 2010; Atalay 2016). In regions such as the eastern part of the Mediterranean, factors affecting changes in land use (e.g. Population and Urban Expansion) result in the degradation of the land (Abu Hammad and Tumeizi 2010; Masoudi et al. 2018), which also applies to Iran and, in particular, the area studied. Determination of the appropriate use of land and preventing further destruction of resources due to population increase should be included in strategies proposed for stable expansion (Bocco et al. 2001; Prato 2007).

By employing GIS and combining the various vector layers of the area, which represent its ecological resources, one can obtain a map showing the most appropriate

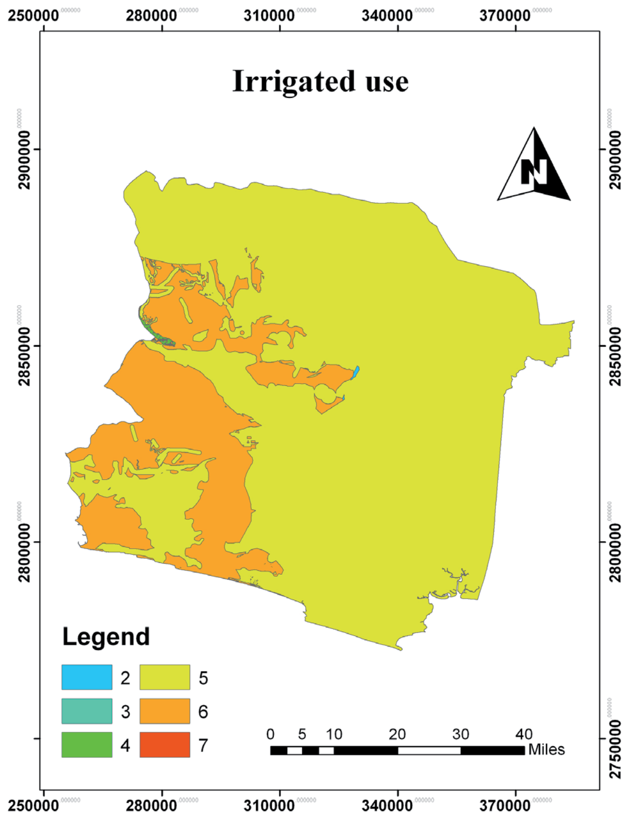

Fig. 3 Land map showing suitability of areas for irrigation agriculture (Note: class 2 → Good to moderate; class 3 → Moderate; class 4 → Mostly moderate; class 5 → Moderate to poor; class 6 → poor; class 7 → unsuitable).

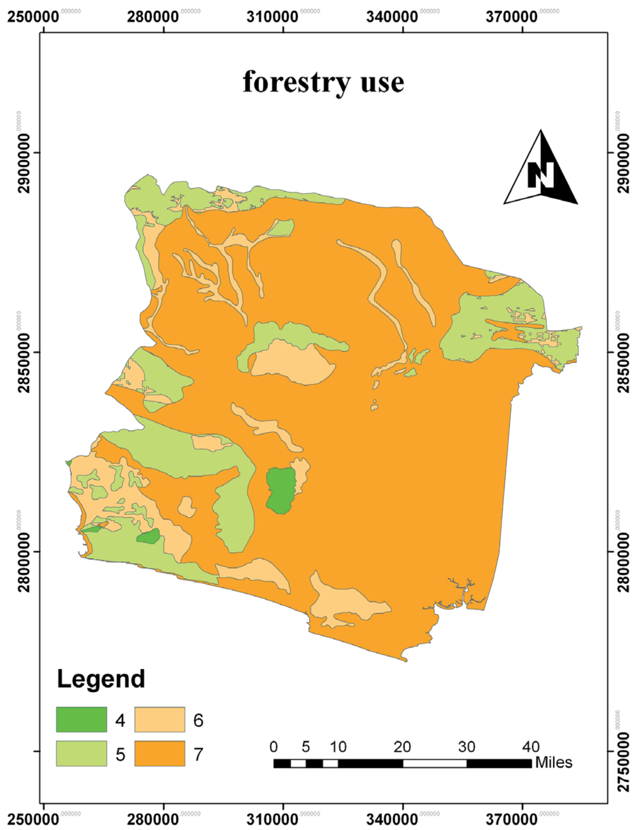

Fig. 5 Land map showing suitability of areas for forestry (Note: class 4 → Mostly moderate; class 5 → poor; classes 6 and 7 → unsuitable).

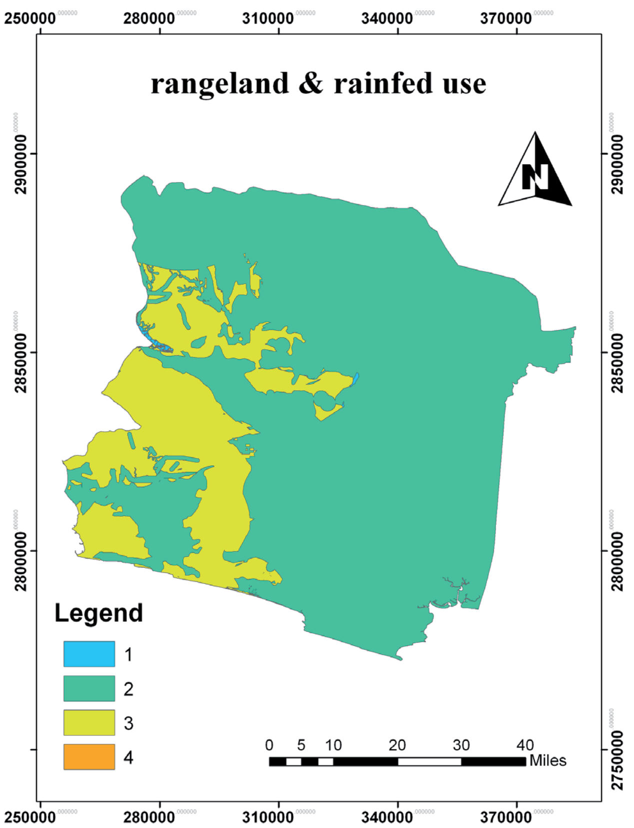

Fig. 4 Land map showing suitability of areas for range management and dry farming (Note: class 1 → Good; class 2 → Moderate; class 3 → Poor; class 4 → unsuitable).

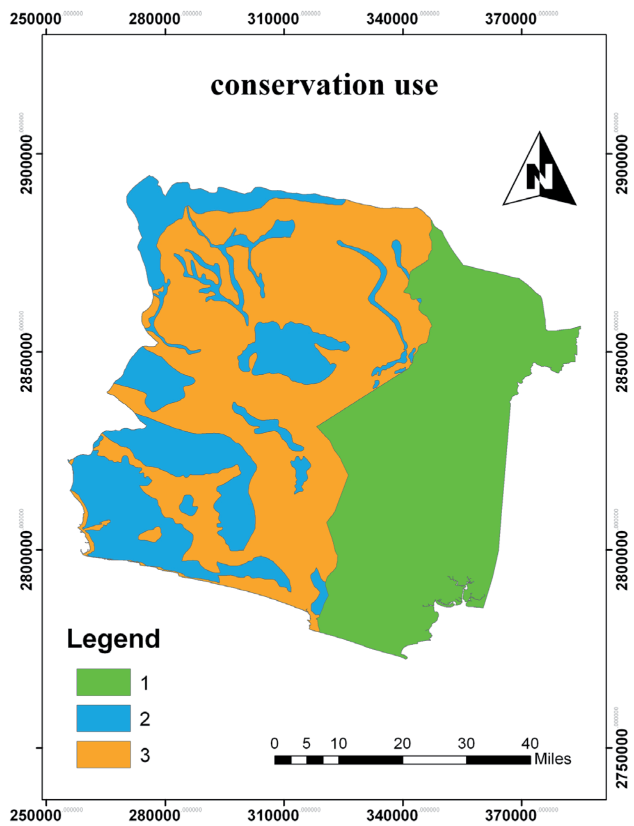

Fig. 6 Land map showing suitability of areas for environmental conservation (Note: class 1 → Good; class 2 → Moderate; class 3 → unsuitable).

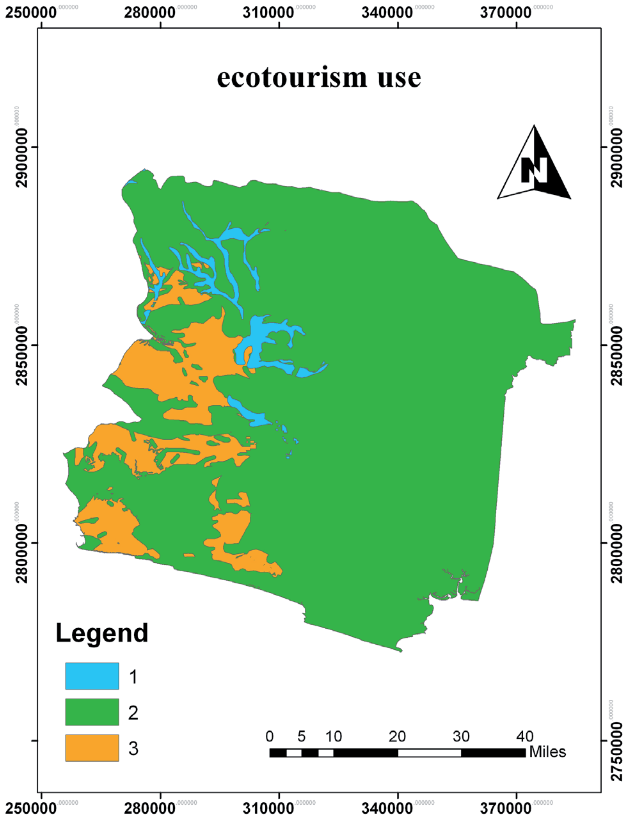

Fig. 7 Land map showing suitability of areas for ecotourism (Note: class 1 → Good; class 2 → Moderate; class 3 → unsuitable).

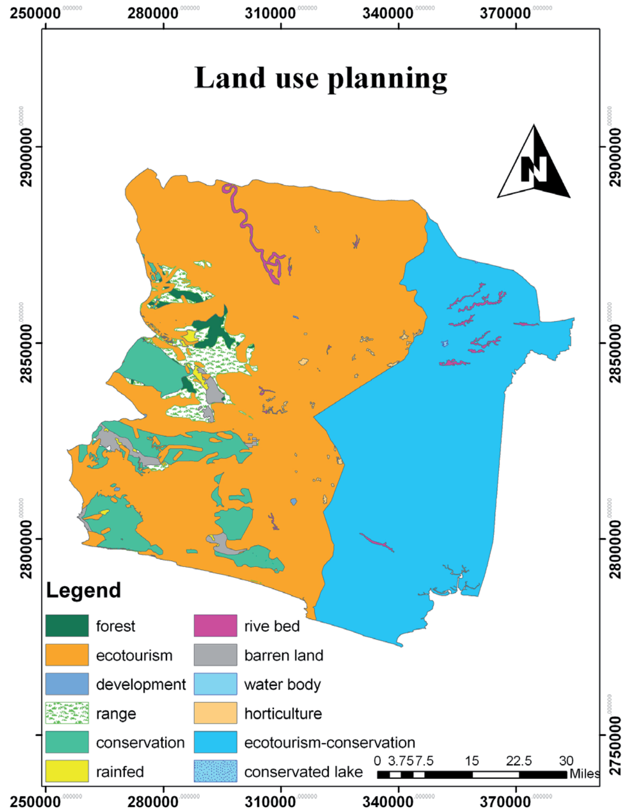

Fig. 9 Land use planning map.

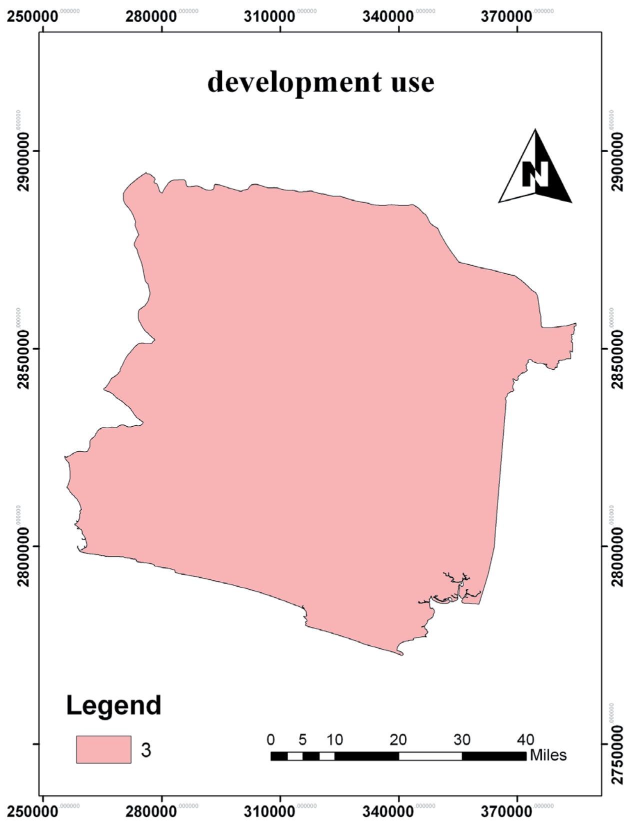

Fig. 8 Land map showing suitability of areas for urban, rural and industrial development (class 3 → poor and unsuitable).

use of the land in an area. However, determination of the priorities for the most appropriate uses of land in these maps must take into consideration the socio-economic conditions in an area or the tendency of residents to utilize the land for certain specific uses.

The capability of areas to sustain particular uses can be reduced by taking into consideration their ecological suitability. This is included in the agricultural and forestry maps with 7 classes, and urban development and ecotourism maps with 3 classes. Use for ecotourism was included because ecotourism is very important in the area studied. Based on the results, the minimum and maximum percentages of area that should be used for development and ecotourism, respectively, can be defined.

The application of Boolean logic to land use evaluation has been criticized by many authors (Burrough et al. 1992; Davidson et al. 1994; Baja et al. 2006; Amiri et al. 2010). In the classic methods like the FAO model for evaluating land use (FAO 1976) the use of maximum limitation make the classification quite rigorous. Because, in Boolean logic, only one index with a lower effect is enough to reduce the suitability of land from highly suitable to unsuitable.

Amiri et al. (2013) utilized methods for assessing the ecological capability of forestry in Dohezar and Sehezar (33, 34), a watershed of Tonekabon city in the Mazandaran Province in Iran. Their findings indicate that the Analytical Hierarchy Process (AHP) and the OWA method was better than other models, even those based on Boolean logic, which is supported by the results presented here.

Babaie-Kafaky et al. (2009) show that if the importance of the multiple-use of Zagros forests is not recognized in the management of this forest it will lose many of its recreational, natural ecosystem characteristics and countless other values.

Examining the land planning maps proved that besides being useful for a single purpose, they can be potential used for many purposes. However, in any one unit, no more than a single type of utilization can ultimately be implemented (Makhdoom 2001). The best use for each unit should be determined by prioritizing the socio-economic conditions in an area and the resident's way of life and their tendency to use land in a specific way. To this end, it is best to consider the following points in prioritizing our findings. In units where there are no socioeconomic limitations, the priority is the one with the highest potential (Espejel et al. 1999). The priority of land use in some of the units is determined based on political needs, and there is no possibility of changing it (Pierce et al. 2005). In some units, where one use has no advantage over another, multiple uses may be proposed (Makhdoom 2001). Generally, current research implemented reforms in Makhdoom's model, which is now more suitable for land use planning. Makhdoom's model and the modified Makhdoom's model have been evaluated in Jahrom and Firuzabad Townships in southern Iran (Asadifard 2015; Masoudi and Jokar 2016; Razaghi 2016). After validation of the two models, the results showed that the modified model was more accurate for land use planning in the areas studied.

Due to the importance of natural hazards, such as drought and climate change, they should be considered in future research. To increase the model's accuracy, methods such as AHP and ANP for Weighting and Fuzzy methodology are also recommended.

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REFERENCES

Abu Hammad A, Tumeizi A (2010) Land degradation: socioeconomic and environmental causes and consequences in the Eastern Mediterranean. Land Degrad Develop 23: 216–226.

Allaouia H, Guoa Y, Choudhary A, Bloemhof (2018) Sustainable agro-food supply chain design using two-stage hybrid multi-objective decision-making approach. Comput Oper Res 89: 369–384.

Amiri MJ, Salman Mahini A, Jalali SGH, Hosseini SM, Azari Dehkordi FA (2010) Comparison of maps overlay systemic method and Boolean-fuzzy logic in the ecological capability evaluation of No. 33 and 34 watershed forests in Northern Iran. Environ Sci 7: 109–124. (in Persian).

Amiri MJ, Salman Mahini A, Jalali SGH, Hosseini SM, Azari Dehkordi FA (2013) OWA Analysis for ecological capability assessment in watersheds. Int J Environ Res 7: 241–254.

Asadifard E (2015) Landuse planning in Firuzabad township based on modifying method of current model using GIS. M.Sc. Thesis, Faculty of Agriculture, Shiraz University. (in Persian).

Atalay E (2016) A New approach to the land capability classification: Case study of Turkey. Procedia Environ Sci 32: 264–274.

Babaie Kafaky S, Mataji A, Ahmadi Sani N (2009) ecological capability assessment for multiple-use in forest areas using GIS-based multiple criteria decision making approach. Am J Environ Sci 5: 714–721.

Baja S, Chapman DM, Dragovich DA (2006) Conceptual model for defining and assessing land management units using a fuzzy modelling approach in GIS environment. Environ Manage 29: 647–661.

Barzani M, Khairulmaini OS (2013) Desertification risk mapping of the Zayandeh Rood Basin in Iran. J Earth Syst Sci 122: 1269–1282.

Biswas A, Baran PB (2005) Application of fuzzy goal programming technique to land use planning in agricultural system. Omega 33: 391–398.

Bocco G, Mendoza M, Velazquez A (2001) Remote sensing and GIS-based regional geomorphological mapping-a tool for land use planning in developing countries. Geomorphology 39: 211–219.

Bojo'rquez-Tapia L, Di'Az-Mondrago NS, Ezcurra E (2001) GIS-based approach for participatory decision-making and land suitability assessment. Int J Geogr Info Sci 15: 129–151.

Burrough PA, MacMallin RA, van Deursen W (1992) Fuzzy classification methods for determining land suitability from soil profile observations and topography. J Soil Sci 43: 193–210.

Cools N, De Pauw E, Deckers J (2003) Towards an integration of conventional land evaluation methods and farmers' soil suitability assessment: a case study in north-western Syria. Agric Ecosyst Environ 95: 327–342.

Davidson DA, Theocharopoulos SP, Bloksma RJA (1994) Land evaluation project in Greece using GIS and based on Boolean and Fuzzy set methodologies. Int J Geogr Inf Syst 8: 369–384.

Espejel I, Fischer DW, Hinojosa A, GarcõÂa C, Leyva C (1999) Land use planning for the Guadalupe Valley, Baja California, Mexico. Landscape Urban Plan 45: 219–232.

Farashi A, Naderi M, Parvin N (2016) Identifying a preservation zone using multi criteria decision analysis. Anim Biodivers Conserv 39: 29–36.

Food and Agriculture Organization of the United Nations (FAO) (1976) A framework for land evaluation. Soils Bulletin 32, Soil resources development and conservation service land and water development division, Rome.

Food and Agriculture Organization of the United Nations (FAO) (1993) Guidelines for land use planning. Soil Resources, Management and Conservation Service, Rome.

Gad A (2015) Land capability classification of some western desert oases, Egypt, using remote sensing and GIS. The Egyptian Journal of Remote Sensing and Space Sciences 18: S9–S18.

Gandasasmita K, Sakamoto K (2007) Practical application of a land resources information system for agricultural landscape planning. Landscape Urban Plan 79: 38–52.

Jafari R, Bakhshandehmehr L (2013) Quantitative mapping and assessment of environmentally sensitive areas to desertification in central Iran. Land Degrad Develop 27: 108–119.

Jozi SA (2010) Evaluation of Ecological Capability using Spatial Multi Criteria Evaluation Method (SMCE) (Case study: Implementation of Indoor Recreation in Varjin Protected Area-Iran). IJESD 1: 273–277.

Makhdoom M (2001) Fundamental of Land Use Planning. Tehran University Press, Tehran. (in Persian).

Masoudi M (2010) Risk assessment and remedial measures of land degradation in parts of Southern Iran. Lambert Academic Publishing (LAP), Germany.

Masoudi M, Jokar P (2016) Suggestion the proposed model of EMOLUP, with new approach in land use planning (step two: prioritizing for different land uses). Environ St 14: 23–36. (in Persian).

Masoudi M, Jokar P, Pradhan B (2018) A new approach for land degradation and desertification assessment using geospatial techniques. Nat Hazards Earth Syst Sci 18: 1133–1140.

National Environment Management Authority (NEMA) (2004) Land use and environment, report on the state of the environment in Kenya. A publication of the National Environment Management Authority (NEMA), Kenya.

Nouri J, Sharifipour R (2004) Ecological capability evaluation of rural development by means of GIS. Iran J Environ Health Sci Eng 1: 81–90.

Oyinloye M, Kufoniyi O (2013) Application of IKONOS satellite images in monitoring of urban land use change in Ikeja, GRA, Lagos, Nigeria. IJESI 2: 1–10.

Pauleit S, Duhme F (2000) GIS Assessment of Munich's urban forest structure for urban planning. J Arboric 26: 133–141.

Peel D, Lloyd M (2007) Neo-traditional planning. Towards a new ethos for land use planning? Land Use Policy 24: 396–403.

Pierce SM, Cowling RM, Knight AT, Lombard AT, Rouget M, Wolf T (2005) Systematic conservation planning products for land use planning: Interpretation for implementation. Biol Conserv 125: 441–458.

Prato T (2007) Evaluating land use plans under uncertainty. Land Use Policy 24: 165–174.

Razaghi S (2016) Assessment and comparison of land use planning in Sepidan region using models of Makhdom, MCE and EMOLUP. M.Sc. Thesis. Faculty of Agriculture. Shiraz University. (in Persian).

Swanson E (2003) Geographic information system (GIS) information enhanced land use planning. Michigan Centre for Geographic Information Department of Information Technology.

Van Lier H (1998) The role of land use planning in sustainable rural systems. Landscape Urban Plan 41: 83–91.

Ward D, Ngairorue BT, Kathena J, Samuels R, Ofran Y (1998) Land degradation is not a necessary outcome of communal pastoralism in arid Namibia. Arid Environ 40: 357–371.

FOREST DEVELOPMENT IN A RESTORED FLOODPLAIN: EFFECTS OF GRAZING, INUNDATION AND VEGETATION

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ABSTRACT

In many countries worldwide, measures have been taken in floodplains for flood prevention and nature rehabilitation. In the Netherlands, floodplains are lowered by excavating in order to enlarge the discharge capacity and create opportunities for the development of river habitats such as forest. As forest can obstruct the flow of water through a floodplain, their development has to be controlled in some cases. In many floodplains, the growth of the vegetation is controlled by cattle and horses. We carried out an exclosure experiment over a twelve-year period in a partly excavated and year-round grazed floodplain along a lowland river in the Netherlands. We focussed on hawthorn (*Crataegus monogyna* Jacq.) as it plays an important role in the obstruction of water flow and in the wood-pasture cycle. Most hawthorn shrubs were recorded on the excavated part of the floodplain with a low cover of tall herbaceous plants. The total number of hawthorn plants was negatively related to inundation on the lower parts of the excavated sites and positively related to inundation on the higher parts of the excavated sites. The herbivores negatively affected the establishment and growth of hawthorn. Although lowering the floodplain by excavation will increase the discharge capacity of the floodplain in the short term, it will decrease in the long term as excavation also increases opportunities for the development floodplain forest. If flood prevention and nature rehabilitation are both goals to be achieved in a floodplain, hawthorn encroachment can be controlled by a clever design and management of grazing.

Keywords: cattle; *Crataegus monogyna*; floodplain forest; hawthorn; horses; wood-pasture hypothesis

Introduction

Natural large rivers are important for nature conservation because of their high biodiversity and their corridor function (e.g. Dynesius and Nilsson 1994; Naiman and Décamps 1997; Hughes et al. 2001; Tockner and Stanford 2002). Large rivers and their floodplains also have high economic value as they are used for shipping, agriculture, industrial activities, urbanization and recreation (e.g. De Waal et al. 1995; Naiman et al. 2002; Tockner and Stanford 2002). In the past few centuries, the large natural rivers and floodplains have dramatically changed due to the building of embankments, canalizations, dams, weirs, groynes, and reclamation of floodplains for agriculture (e.g. Dynesius and Nilsson 1994; Rosenberg et al. 2000; Tockner and Stanford 2002; Bunting et al. 2013). As a consequence, natural floodplains have become endangered landscapes, and many types of riverine habitats such as floodplain forests have become rare (e.g. Dynesius and Nilsson 1994; Brown et al. 1997; Olson and Dinerstein 1998; Tockner and Stanford 2002; Bunting et al. 2013). Restoration and conservation of these floodplain forests has become an important goal of river management (e.g. Brown et al. 1997; Leyer et al. 2012; Bunting et al. 2013).

However, floodplain forest can seriously obstruct water flow and reduce the discharge capacity of rivers, resulting in high flood water levels and associated safety risks (Makaske et al. 2011; Leyer et al. 2012). In order to achieve the safety goals, control of establishment and growth of woody species is sometimes needed. Especially of thorny shrubs such as hawthorn (*C. monogyna*), as they have the highest hydraulic roughness of the different types of floodplain vegetation (Van Velzen et al. 2003).

Hawthorn is a shrub or small tree, up to 10 m, which is a common woody plant in Western and Central Europe. Hawthorn flowers (white, hermaphroditic, one style, 5–25 stamens) in April-June (Garcia and Chacoff 2007). Although the species is partially self-compatible, it relies on visits from insects to set fruit. It produces many single seeded fruits (red berries) in summer, which are ripe in September-October. The fruits are eaten by birds and mammals, which excrete the undigested seeds and spread the seeds (Herrera 1984; Snow and Snow 1988). To germinate, seeds need light and bare mineral soil as they do not establish in closed vegetation with a litter layer (Watt 1934; Smith 1980; Buttenschøn and Buttenschøn 1985). Hawthorn establishes on moist to dry sandy to clayey soils and can endure some inundation (Vreugdenhil et al. 2006). On its twigs and branches, Hawthorn produces thorns 1–3 cm long to protect itself from browsing by large herbivores (Gill 2006; Hester et al. 2006).

In many conservation areas in the Dutch floodplains, year-round grazing with low numbers of cattle and horses (<0.4 per ha; Kuiters et al. 2003) is used as a management tool to control the growth of vegetation. Some

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shrub and tree species such as *Salix* spp. or *Populus* spp. can easily be suppressed by large herbivores (Van Splunder 1998; Baraza et al. 2006; Cornelissen et al. 2014a, 2014b), but others, like hawthorn, are relatively resistant to grazing because of its thorns (Gill 2006; Hester et al. 2006). Because of their physical defence, hawthorn and other thorny shrubs also play an important role in the woodland-grassland cycle of the wood-pasture hypothesis of Vera (2000). According to this hypothesis, large herbivores are responsible for the development of park-like landscapes. They contribute to the transition of woodland to grassland by debarking trees, maintain short grazed grasslands and create opportunities for the re-establishment of thorny shrubs in these grazed grasslands. Once established in grazed grasslands, hawthorn can serve as refugium by protecting other, more palatable tree species (Baraza et al. 2006; Gill 2006) and initiate forest development.

In order to optimize both safety and ecological values, it is important to monitor encroachment by woody plants after measures taken to restore safety and ecology are realized and grazing has started. In general, establishment and growth of shrubs and trees on floodplains largely depend on flooding, substrate, light and root competition with grasses and tall herbaceous plants, and herbivores, especially during the early life stages of the woody species (Jones et al. 1989; Streng et al. 1989; Siebel 1998; Van Splunder 1998; Hughes et al. 2001; Vreugdenhil et al. 2006).

In order to understand the effect of grazing, inundation and vegetation on the encroachment by woody plants, we carried out an exclosure experiment over a twelve-year period in a restored and year-round grazed floodplain along the river Waal in the Netherlands. We focused on hawthorn because of its high flow resistance and its role in the wood-pasture hypothesis. This shrub is less flood tolerant than softwood species (Vreugdenhil et al. 2006). Hawthorn germinates on bare soil and in grazed grasslands, but survival of seedlings is low in shaded environments (Watt 1934). Hawthorn can resist grazing pressure better than non-thorny softwood species (Good et al. 1990; Baraza et al. 2006). We expected that: (1) in the excavated areas with bare sandy soils and low cover of tall herbaceous plants, more hawthorn would become established than in the non-excavated, vegetated areas with high cover of tall herbaceous plants; (2) establishment of hawthorn is negatively related to inundation; (3) cattle and horses affect the establishment and growth of hawthorn; (4) in the grazed grasslands there will be more hawthorn than in the ungrazed grasslands.

Material and Methods

Study area

This study was carried out on the floodplain Afferdense and Deestse Waarden (51°53´44´´N; 5°37´40´´E) along the river Waal in the Netherlands. The study area was approximately 46 ha. In 1996, 23 ha of this area, originally covered with grasslands, were excavated and the first section of a new side channel was dug within the lowered floodplain to increase the discharge capacity and create opportunities for the development of nature (Fig. 1).

The unexcavated parts of the study area consisted of nutrient rich clayey soils and the excavated parts of nutrient poor sandy soils (Table 1). Water levels of the river Waal at the study area varied within and among years (Fig. 2). High levels were recorded during winter and low levels during summer. Average water levels were higher during 1999–2003 and lower during 1996–1998 and 2003–2007.

On nutrient-rich soil the vegetation consisted mainly of short grasslands (e.g. *Lolium perenne* L., *Poa trivialis* L., *Festuca rubra* L., *Trifolium repens* L., *Potentilla reptans* L., *Taraxacum* spp., *Cirsium arvense* (L.) Scop., *Urtica di-*

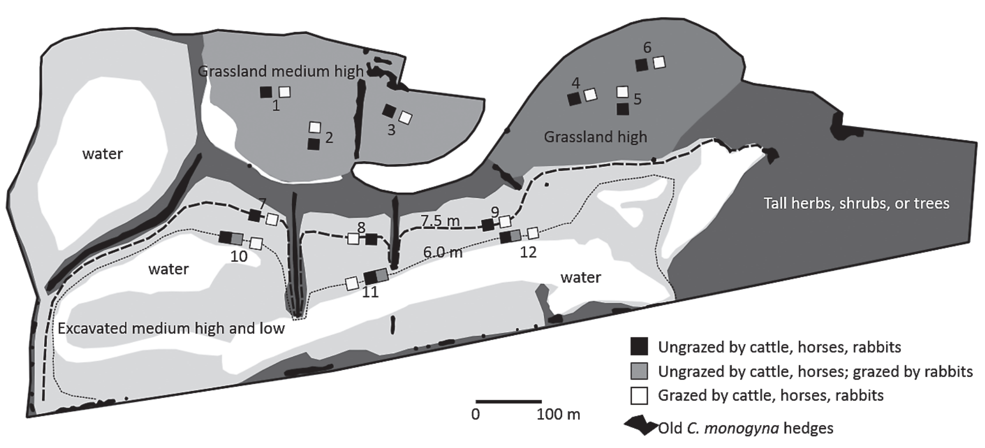

Fig. 1 Location of the strata grassland high, grassland medium high, excavated medium high, excavated low, and blocks (nr 1–12) in the study area. The blocks consist of grazed and ungrazed plots indicated by the black, grey and white squares. The dashed lines in the excavated part of the floodplain indicate the elevation levels of 6.0 m (low) and 7.5 m (medium high) + NAP.

Table 1 Characteristics strata study area. Inundation characteristics are averages and standard errors of mean (within parentheses) over the period 1996–2007. NAP is the Dutch reference level for elevation, which is about sea level. January–December and March–October indicate the period from January to December and from March to October.

		Strata			
		Not excavated		Excavated	
		High	Medium high	Medium high	Low
Vegetation 1996		Grassland	Grassland	Bare soil	Bare soil
Soil (% lutum (< 2μm))		26	25	4	3
Ground level (m + NAP)		9.0	7.5	7.5	6.0
Total inundation (days)	January–December	7 (2)	27 (6)	27 (6)	83 (13)
Inundation frequency (N)	March–October	3 (1)	12 (4)	12 (4)	42 (10)
	January–December	1 (<1)	3 (1)	3 (1)	7 (1)
Average length of inundation events (days)	March–October	1 (<1)	1 (<1)	1 (<1)	4 (1)
	January–December	5 (1)	8 (1)	8 (1)	14 (2)
Average water depth during inundation (cm)	March–October	3 (1)	5 (1)	5 (1)	12 (3)
	January–December	16 (6)	93 (11)	93 (11)	115 (9)
	March–October	5 (4)	65 (15)	65 (15)	88 (12)

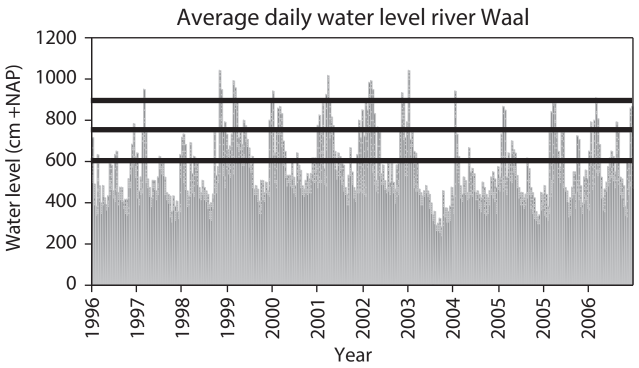

Fig. 2 Average daily water level of the river Waal at Afferdense and Deestse Waarden. The horizontal lines show the surface levels of the different strata of 6.0, 7.5 and 9.0 m + NAP.

oica L.; 12 ha), and a mosaic of tall herbaceous plants, tall grasses and willows (e.g. *C. arvense*, *U. dioica*, *Symphytum officinale* L., *Phalaris arundiancea*, L. *Glyceria maxima* (Hartm.) Holmb., *Salix* spp.; 11 ha). In the excavated areas, vegetation was almost absent during the first year. In the second year, pioneer species (e.g. *Chenopodium album* L., *Erigeron canadensis* (L.) Cronq., *Matricaria maritima* (L.) W.D.J.Koch, *Rumex maritimus* L.) colonized the area. Within the study area, some old hawthorn shrubs and hedges were present. These were remnants of the former agricultural landscape.

Before 1996, the study area was grazed by high numbers (1–2 animals/ha) of dairy cattle, and parts of the grassland that were invaded by tall herbaceous plants such as *C. arvense* or *U. dioica,* were mown. After 1996, the study area was grazed during summer (April–November) by privately owned cattle (c. 15 cows) and horses (c. 20 mares). During winter (November–March), the owner moved the cattle to the farm for supplementary feeding and shelter, but the horses remained in the area. The horses did not get supplementary feeding and the grasslands were not mown anymore.

Exclosure experiment

We carried out an exclosure experiment to determine the effects of herbivores, vegetation and inundation on establishment and growth of woody species. The experiment consisted of 12 blocks (i.e. experimental unit; Hurlbert 1984) to test the effect of herbivores. Each block consisted of an ungrazed (exclosure) and a grazed plot (both 15 × 15 m). The grazed plot was randomly placed at a distance of 10 m from the exclosure. All exclosures were made to exclude not only cattle and horses but also small herbivores such as rabbits and hares.

Based on ground level and excavation, we distinguished four different strata: (1) excavated low; (2) excavated medium high; (3) grassland medium high; (4) grassland high (Fig. 1, Table 1). The strata grassland low and excavated high were not present in the study area. Although the strata are not replicated, as replication was not possible at this scale, we used these strata in our experiment to determine the effects of inundation and vegetation, next to herbivores, on the establishment and growth of woody species on this floodplain. To describe the effect of excavation and grazing by all herbivores on vegetation and hawthorn, we compared the stratum grassland medium high with excavated medium high. To determine the effect of inundation and grazing by all herbivores on vegetation and hawthorn, we compared the stratum excavated medium high with excavated low. The stratum grassland high was not used in these tests as no hawthorn became established on this stratum. To analyse these effects, in each stratum 3 blocks were randomly placed after the excavation in the summer of 1996.

In the stratum “excavated low”, we expected the establishment of high numbers of *Salix* spp. and a rapid increase in cover and height of these species immediately after excavation (Van Splunder 1998). To investigate the effect of small herbivores on these fast colonizing, palatable softwood species, we added an extra exclosure to the blocks in this stratum, excluding only cattle and horses.

For describing the results, we used the following codes for the strata:

	Grassland	Excavated
High (9.0 m NAP)	GH	
Medium low (7.5 m NAP)	GM	EM
Low (6.0 m NAP)		EL

To distinguish between grazed and ungrazed sites within the strata, we added to these codes the letter -g or -u, respectively.

Inundation

River water level data were obtained from the Ministry of Infrastructure and Water management. We determined total number of days with inundation, number of inundation events (frequency), average length of inundation events and average water depth during inundation as they explained most of the variation in establishment and growth in other studies (e.g. Siebel 1998; Van Splunder 1998; Vreugdenhil et al. 2006). We also distinguished two periods of inundation: 1) from January to December and 2) from March to October (growing season).

Herbivore numbers and daily energy expenditure

The number of cattle and horses stayed the same over the years from 1996 to 2007 (see section study area). We counted the numbers of hares and rabbits during evenings in winter (December–January) using a light. In 1997, 1999 and 2007, eight counts per winter were carried out along a fixed route through the whole of the area studied. There were no roe deer (*Capreolus capreolus* L.) or beaver (*Castor fiber* L.) on this flood plain (pers. comm. State Forestry Service).

To compare the effect of grazing by large and small herbivores and to compare our results with that of others (e.g. Bakker et al. 2004), we transformed herbivore densities into daily energy expenditure (DEE). According to Bakker et al. (2004), the amount of energy an average animal spends daily is two times its basal metabolic rate (BMR): DEE = $2 \times 2930 \times W^{0.75}$ kJ per day. For cows and mares of different ages (1–15 years old) we assumed an average weight of 350 kg based on Cornelissen et al. (1995), for rabbits we used an average weight of 1.5 kg (Wallage-Drees 1988) and for hares 4 kg (Lange et al. 1994).

Vegetation surveys

In August 1997, 1999, 2001 and 2007, we assessed vegetation cover and height of two structural layers: (1) low grasses and low herbaceous plants; (2) tall herbaceous plants. We established four permanent quadrats of 2 × 2 m within the ungrazed plot at 2 m distance from the fence of the exclosure, and in the same way within the grazed plot. Cover was estimated (vertical projection on the ground in percentages) and height was measured with a ruler and a polystyrene disk (radius 50 cm, weight 320 g), which was lowered over a ruler on to the sward. Average cover and height were calculated for each ungrazed and grazed plot. These averages were used to calculate averages and standard errors of cover and height of grazed and ungrazed vegetation for each stratum.

In August 2007, cover and height of all woody species were assessed in the total area (15 × 15 m) of the ungrazed and grazed plots. Cover was estimated visually as a percentage of the area of ground occupied. Heights of woody plants were measured with a ruler. The measurements per ungrazed and grazed plot were used to calculate averages and standard errors of cover and height of woody species in grazed and ungrazed plots per stratum.

In November 2007, all hawthorn plants present in the ungrazed and grazed plots were harvested for age determination in order to investigate the effect of inundation, vegetation and grazing on establishment and growth. Height, crown diameter (mean of maximum and minimum diameter), stem circumference and total number of twigs (up to 2 m height) were measured before the destructive harvest. Circumference was later transformed into an average stem diameter, as it is an important parameter for determining the hydraulic roughness of the shrub.

The nomenclature used for plant species follows that of Van der Meijden (2005).

Age determinations of hawthorn

Age determination of hawthorn was based on growth rings and was carried out as described by Decuyper et al. (2014).

Statistical analysis

We used Generalized Linear Models with a Poisson distribution and a log-link function to test the effects of ground level (medium high or low), excavation (yes or no) and herbivores (yes or no) on total number of hawthorn present in 2007; this revealed no over dispersion. We also nested block within excavation or ground level to correct for possible random variation among blocks.

To test the effect of grazing by rabbits on the total number of hawthorn present in 2007, we compared the ungrazed and grazed plots in the stratum excavated low. We used Generalized Linear Models with a negative binomial distribution and a log-link function to test these effects because of over dispersion. Grazing (yes or no) was used as predictor. Block was also incorporated to correct for possible random variation among blocks.

General Linear Model Repeated Measures was used to test the effects of excavation or ground level, herbivores (between-subjects factors) and year (within-subjects factor) on the dependent variables cover and height of low grasses and herbaceous plants and tall herbaceous plants. We nested block within excavation or ground level to correct for possible random variation among blocks. To meet the assumptions of the statistical test, data in percentages were arcsine transformed (Sokal and Rohlf 1981).

General Linear Model Univariate procedure was used to test differences between slopes or intercepts of the different relations between inundation and established hawthorn or hawthorn growth. Non-linear relations were transformed in order to obtain linear relationships before testing differences between slopes or intercepts.

All data were analysed using SPSS for Windows version 23 (Norusis 1996). All error bars in graphs are Standard Errors of Means (SEM).

Results

Herbivore numbers and DEE

Horses grazed the area year-round in the same numbers every year: 20 mares. Cattle grazed the area from April until November in the same numbers every year: 15 cows. During the winters of 1997/1998, 1999/2000 and 2007/2008, average rabbit numbers within the study area were respectively 35, 25 and 90. Almost nearly no hares were recorded (average <1).

During summer, DEE per day of the 35 large herbivores was about 1650 MJ for the whole study area, and during winter DEE was about 950 MJ. DEE of rabbits varied between 20 and 70 MJ for the whole study area. During winter, total DEE of mares was about 15–50 times higher than that of rabbits. As DEE of the large herbivores almost doubled during summer, the difference between large and small herbivores probably will have been even greater during that period.

Established woody species

The results of the survey in 2007 (Table 2) reflect the net effect of establishment and mortality and the net effect of growth and losses over 12 years. In general, there were fewer species of woody plants on the grasslands than on the excavated strata. Within the grasslands, there were no woody species at all on GH. On the excavated substrates, there were more thorny shrubs on EM and more *Salix* spp. on EL. Heights of woody species varied among strata and between grazed and ungrazed.

Effects of rabbits

Total number of hawthorn recorded in 2007 in the plots grazed by rabbits (Fig. 3) was not different from that in ungrazed plots (P = 0.9882). Growth parameters of hawthorn in the plots grazed by rabbits were not different from those recorded in ungrazed plots (Fig. 5;

Table 2 Average cover and height of woody species in 2007. SEM is given in parentheses. C = cattle; H = horses; R = rabbits.

Strata									
Habitat	**Grassland**				**Excavated**				
Ground level	**High**		**Medium high**		**Medium high**		**Low**		
Grazed/ungrazed herbivores	**Ungrazed**	**Grazed CHR**	**Ungrazed**	**Grazed CHR**	**Ungrazed**	**Grazed CHR**	**Ungrazed**	**Grazed CHR**	**Grazed R**
Species	**Cover (%)**								
Crataegus monogyna	0	0	5 (5)	2 (1)	15 (6)	2 (1)	3 (2)	0	3 (2)
Rosa canina	0	0	0	0	9 (6)	7 (3)	1 (<1)	0	1 (<1)
Rubus ceasius	0	0	19 (18)	0	7 (3)	1 (1)	4 (<1)	0	3 (1)
Cornus sanguinea	0	0	0	0	<1 (<1)	0	0	0	0
Sorbus aucuparia	0	0	0	0	0	0	<1 (<1)	0	0
Fraxinus excelsior	0	0	0	0	0	0	0	0	<1 (<1)
Populus nigra	0	0	0	0	0	0	<1 (<1)	0	1 (1)
Salix spp.	0	0	0	0	10 (5)	0	40 (15)	3 (1)	32 (9)
Total woody cover	0	0	21 (20)	2 (2)	28 (9)	9 (3)	43 (16)	3 (1)	34 (11)
	Height (cm)								
Crataegus monogyna			450 (–)	412 (–)	120 (20)	78 (26)	47 (125)		77(27)
Rosa canina					150 (29)	147 (29)	83 (13)		105(18)
Rubus ceasius			85 (5)		37 (13)	20 (–)	53 (12)		53(12)
Cornus sanguinea					65 (–)				
Sorbus aucuparia							60 (–)		
Fraxinus excelsior									20 (–)
Populus nigra							390 (–)		275(–)
Salix spp.					300 (50)		483 (17)	12 (2)	427(18)

Table 3 P-values Generalized Linear Model with Poisson distribution and log-link for total number of established hawthorn in 2007. G = grazing (yes or no); E = elevation (7.5 m or 6.0 m) or excavation (yes or no); G × E = interaction effect; Block was nested within the factor Elevation or Excavation to correct for possible random variation among blocks. The bold p-values highlight that the effects are significant.

	G	E		G × E	Block (E)
		Ground level	Excavation		
Grassland 7.5 m vs Excavated 7.5 m	**0.0006**		**0.0001**	0.2344	<0.0001
Excavated 7.5 m vs Excavated 6.0 m	**<0.0001**	**<0.0001**		a	<0.0001

[a] Unable to compute due to absence of hawthorn in grazed excavated 6.0 m.

Table 4 P-values General Linear Model Repeated Measures for the effects of grazing and elevation or excavation on cover and height of low grasses and herbs and tall herbs. **A**, **B** and **C** show the results of the comparisons between different strata. Grazing = grazed or ungrazed by cattle, horses and rabbits. Elevation = 9.0 m vs 7.5 m or 7.5 m vs 6.0 m. Excavation = yes or no. Year = 1997, 1999, 2001, 2007. **D** shows the results of the comparison between plots grazed by rabbits and ungrazed plots on the stratum excavated low.

	Grasses and herbaceous plants		Tall herbaceous plants	
	Cover	Height	Cover	Height
A. Grassland 9.0 m vs Grassland 7.5 m				
Test of Between-Subjects Effects				
Grazing	0.4770	**0.0005**	**0.0170**	**0.0225**
Ground level	0.9207	0.4264	0.7119	0.2218
Grazing x Ground level	0.8899	0.2662	**0.0267**	0.4567
Block (within Ground level)	0.7144	0.9951	0.2132	0.3905
Test of Within-Subjects Effect				
Year	**0.0016**	**<0.0001**	**0.0001**	**<0.0001**
Year x Grazing	**<0.0001**	**<0.0001**	0.3875	**0.0105**
Year x Ground level	0.9627	0.3046	0.0900	0.7185
Year x Grazing x Ground level	0.8761	0.9555	0.6587	0.2826
B. Grassland 7.5 m vs Excavated 7.5 m				
Test of Between-Subjects Effects				
Grazing	**0.0495**	**0.0083**	0.8015	**0.0233**
Ground level	**0.0065**	**0.0436**	**0.0262**	**0.0489**
Grazing x Ground level	0.1543	**0.0318**	0.5611	0.1062
Block (within Ground level)	0.8496	0.9300	0.3488	0.4961
Test of Within-Subjects Effect				
Year	**<0.0001**	0.1676	**0.0060**	**0.0001**
Year x Grazing	**0.0003**	**0.0241**	0.7302	**0.0324**
Year x Ground level	**0.0002**	0.0602	**0.0023**	0.1371
Year x Grazing x Excavation	**0.0022**	**0.0336**	0.7909	0.6087
C. Excavated 7.5 m vs Excavated 6.0 m				
Test of Between-Subjects Effects				
Grazing	**0.0174**	0.3408	0.9297	**0.0103**
Ground level	**0.0087**	**0.0404**	0.2063	**0.0387**
Grazing x Ground level	0.4235	0.5569	0.3179	0.0577
Block (within Ground level)	0.5449	0.9099	0.3378	0.4482
Test of Within-Subjects Effect				
Year	**<0.0001**	0.7767	0.1837	**0.0086**
Year x Grazing	0.1613	0.1971	0.4845	0.0734
Year x Ground level	**0.0057**	0.8392	0.3483	0.2544
Year x Grazing x Ground level	0.1359	0.4527	0.4091	0.0634
D. Excavated 6.0 m: Grazed by Rabbits vs Ungrazed				
Test of Between-Subjects Effects				
Grazing	0.3669	0.6077	0.4586	0.0657
Block	0.3889	0.9157	0.6732	0.0626
Test of Within-Subjects Effects				
Year	0.1503	0.0832	0.2286	0.1475
Year x Grazing	0.3470	0.4698	0.1414	0.4078

Table 5 P-values General Linear Model Univariate for testing differences in slopes and intercepts of regression lines between strata and between grazed and ungrazed (see Fig. 5). CHR = cattle, horses and rabbits. R = rabbits. The bold P-values highlight that the differences are significant.

	Excavated medium high vs excavated low for ungrazed CHR		Ungrazed CHR vs Grazed CHR on excavated medium high		Ungrazed CHR vs Grazed R on excavated low	
	Slopes	Intercepts	Slopes	Intercepts	Slopes	Intercepts
Height	0.4633	**<0.0001**	0.5355	**0.0159**	0.8290	0.9007
Crown	0.7795	0.7965	0.1495	0.4985	0.8167	0.2819
Stem	0.1001	0.5687	0.4353	0.1097	0.2806	0.3595
Twigs	0.6137	0.0771	0.2380	0.8725	0.7902	0.9641

Table 5). Cover and height of low grasses and herbaceous plants and of tall herbaceous plants in plots grazed by rabbits (Fig. 6) were not different from that recorded in ungrazed plots (Table 4).

Effects of excavation, inundation and large herbivore grazing

The total number of hawthorn recorded over the period 1996–2007 was affected by herbivores and ground level or excavation and was highest on EM-u and absent on GH and EL-g (Fig. 3, Table 3). The years in which hawthorn were recorded differed among strata (Fig. 3). On GM, hawthorn were only present during the first three years. On EM, they were present almost throughout the whole period, but the highest numbers were recorded between 1998–2002. On EL-u, most were recorded between 2002–2005.

Establishment on the excavated strata was related to water level. In general, more hawthorn were recorded on the less inundated EM than on the more inundated EL, indicating a negative relation with inundation (Fig. 3, Table 3). However, if we look at the results in detail, numbers on EM was positively related to inundation whereas on EL it was negatively related (Fig. 4). In the grazed excavated strata, relationships were the same as in the ungrazed strata.

Of the growth parameters only height was affected (Fig. 5). On EM-u, shrubs were 50–100 cm shorter than on EL-u (comparison of the heights of the same age classes). On EM-u, shrubs were 25–50 cm taller than on EM-g.

Cover and height of the grass and herbaceous plant layers differed between strata, years and between grazed and ungrazed sites (Fig. 6; Table 4). The cover of low grasses and herbaceous plants decreased in the grasslands and increased on the excavated strata. On the grasslands, the decrease in cover was greater at the ungrazed than at the grazed sites. On the excavated strata, the increase in cover was lower at the ungrazed than at the grazed sites. The height of the low grasses and herbaceous plants was greater in the grasslands than in the excavated strata and also greater at ungrazed than at grazed sites.

The cover and height of tall herbaceous plants increased strongly on the grasslands. On the excavated strata, cover was stable and height increased.

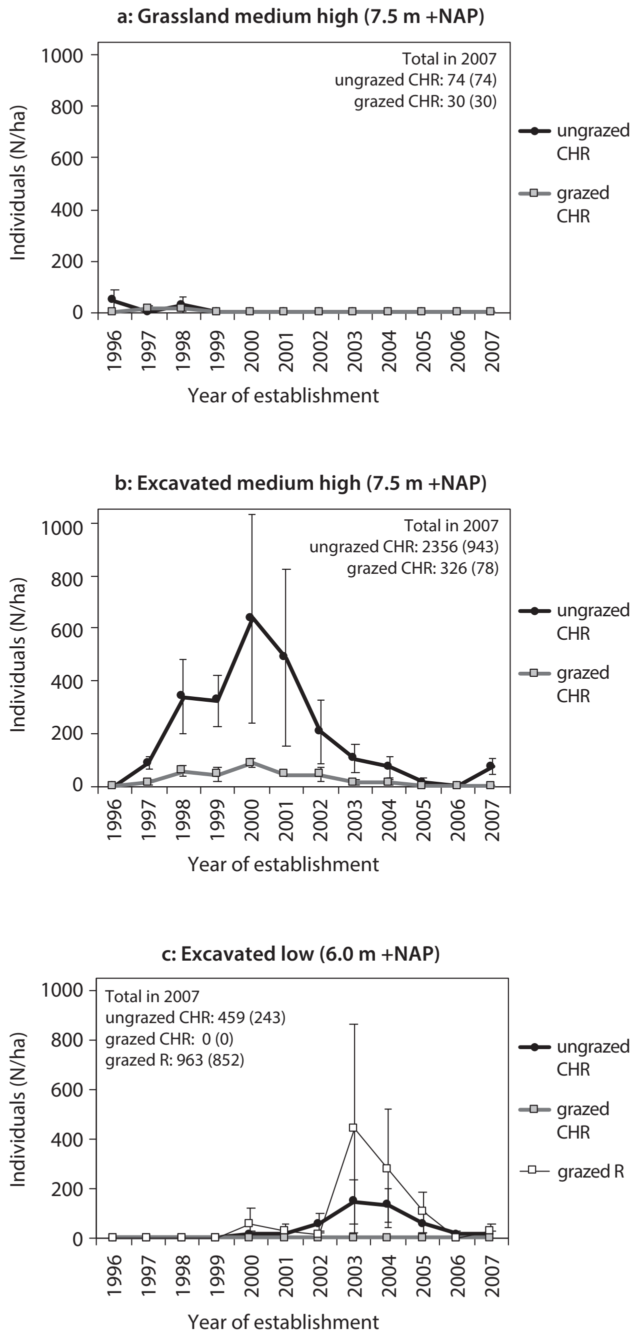

Fig. 3 Number of hawthorn recorded on different strata in different years. C = cattle, H = horses, R = rabbits. In the corner of the graphs the average number of hawthorn recorded over the period 1996–2007, is given; SEM within parentheses.

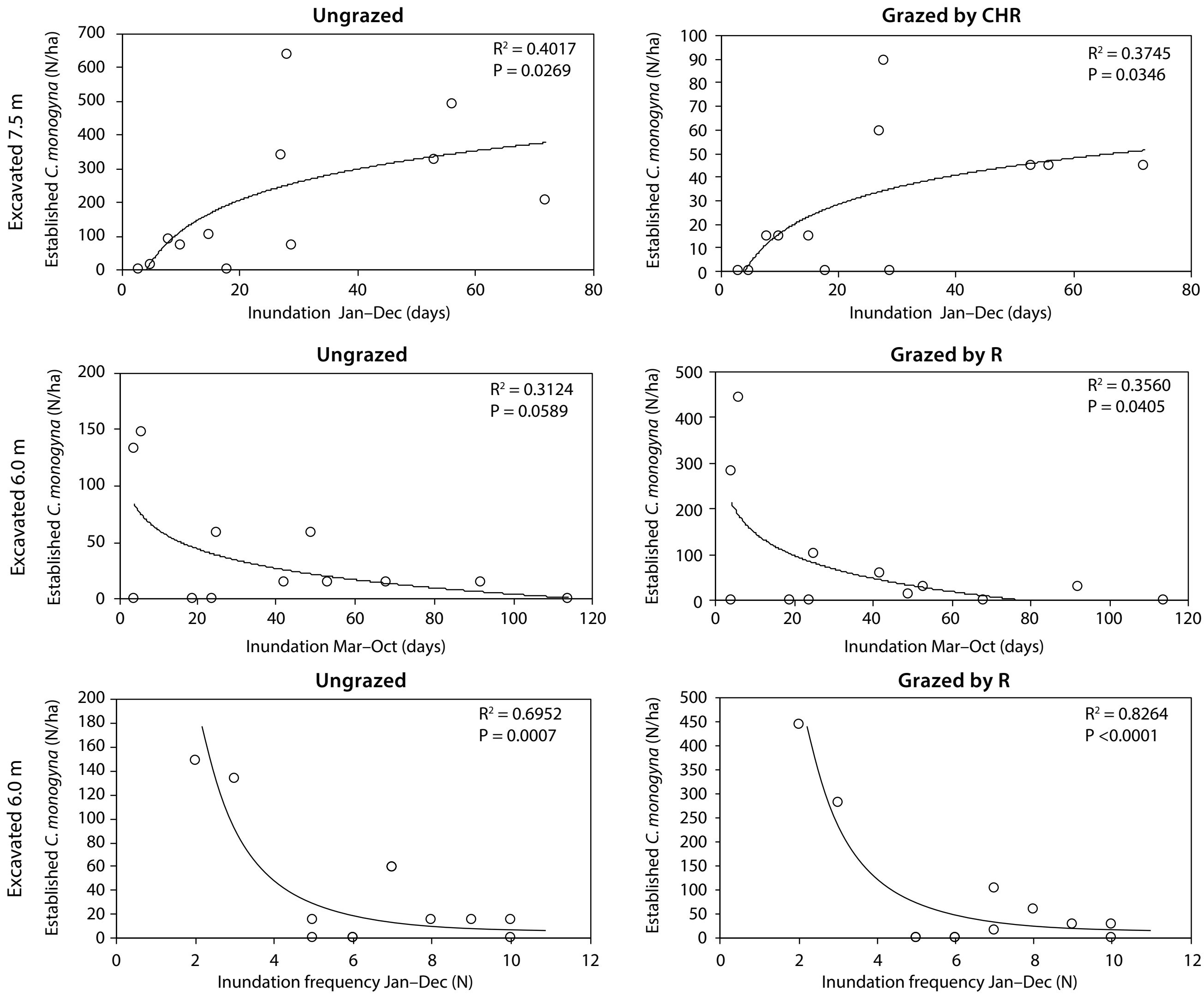

Fig. 4 Relations between inundation and establishment of hawthorn (top and middle) and between inundation frequency and establishment of hawthorn (bottom) on ungrazed and grazed strata. Establishment of hawthorn during the period 1996–2007. C = cattle, H = horses, R = rabbits. Note differences in Y- and X-axes.

Discussion

Effects of excavation

This study revealed that in the excavated areas of the floodplain there was a greater establishment of hawthorn than in the non-excavated grasslands, which supports our first expectation. There were also more other woody species present on the excavated areas than on the grasslands. After excavation, bare substrate was exposed over large areas for a few years, as the fertile soil layer was removed. The development of tall herbaceous vegetation was much less than in the grasslands, providing more opportunities for the establishment of hawthorn and other woody species. Similar effects of vegetation cover on the establishment of woody species are reported in other studies (e.g. Siebel 1998; Bokdam and Gleichman 2000; Niinemets and Valladares 2006). In the non-excavated grasslands, the woody seedlings have to compete with tall herbaceous plants such as *C. arvense* and *U. dioica* for light and nutrients. These fast growing tall herbaceous plants are stronger competitors than the seedlings of woody species in highly productive environments, such as the grasslands on clayey soils. We conclude that the tall herbaceous vegetation played an important role in the establishment of hawthorn and the other woody species.

This detailed study of hawthorn revealed that more shrubs established on EM than on EL. This supports our second expectation that establishment is negatively affected by inundation. However, when the results are examined in more detail, the negative relation was true for the EL sites, but not for the EM sites, where the relation was positive. We assume that on EL and during periods of high water level, inundation was sufficiently frequent, to prevent seeds from germinating and seedlings becoming established as frequent inundation results in the seedlings due to drowning or oxygen deprivation (Niinemets and Valladares 2006; Vreugdenhil et al. 2006). Only during periods with relatively low water levels (2003–2007), hawthorn colonized and survived on EL. The less frequent inundation of EM probably resulted in the high-

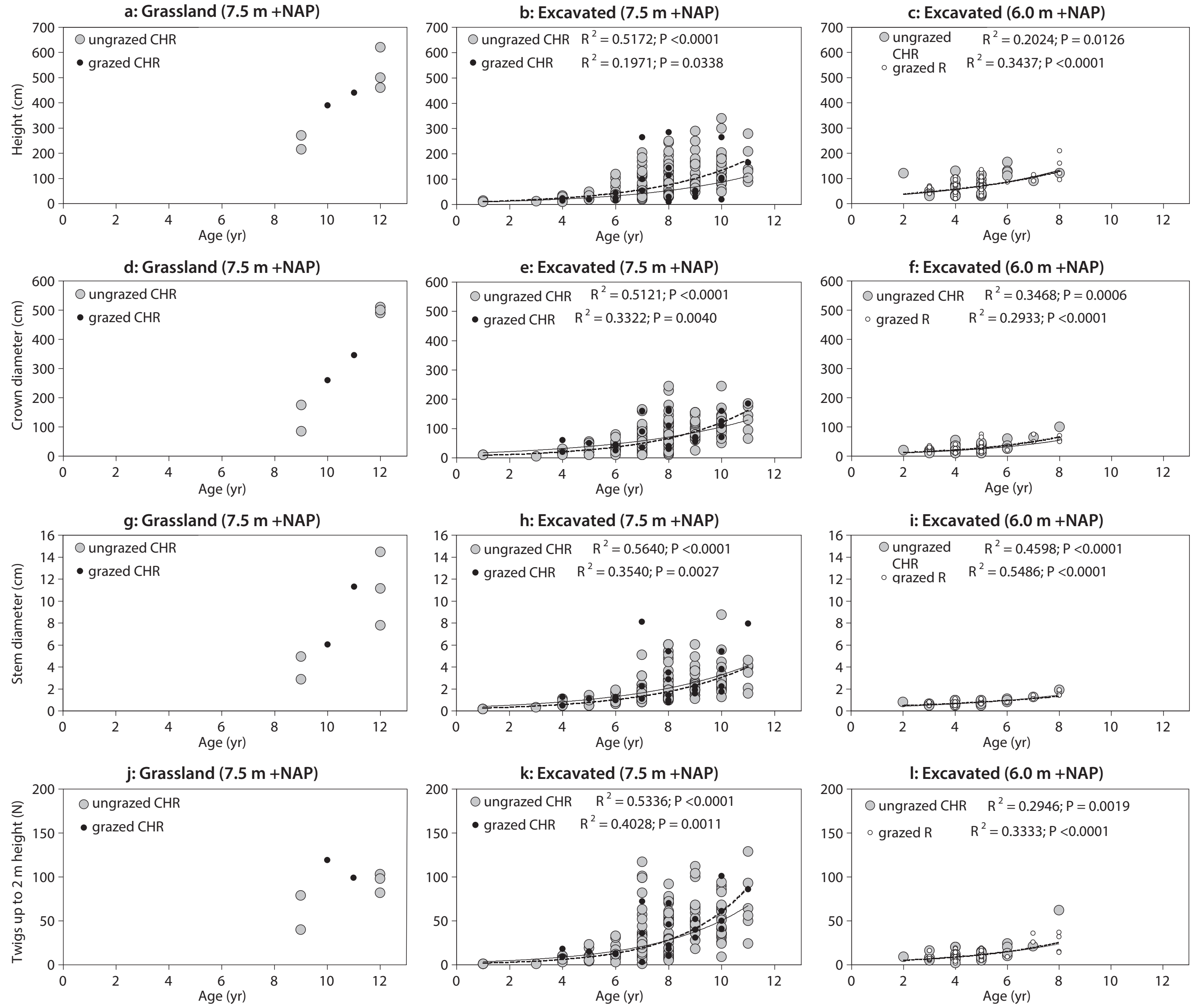

Fig. 5 Height (A–C), crown diameter (D–F), stem diameter (G–I) and number of twigs (J–L) of hawthorn of different ages growing on different strata and in grazed and ungrazed situations. C = cattle, H = horses, R = rabbits. Within the graphs R^2 and P-values are given for the relations. See table A (below) for results of the tests for differences between relations.

er survival of hawthorn there than on EL, but this does not explain the positive relation between inundation and establishment. A potential explanation of this positive relation could be fruit dispersal by water. During field observations (pers. obs. P. Cornelissen), fruit were found in drift line material. As fruit of hawthorn can also be dispersed by water, lower water levels can lead to less or no seed deposition by water on the higher ground. Although hawthorn fruit can also be dispersed by wind (very short distances), birds and mammals (short and long distances; Good et al. 1990; Martinez et al. 2008), only water can disperse large amounts of fruit over long distances.

On GM, a similar positive relation between inundation and seed deposition exists. The effect, however, is different from that on EM. The difference may be explained by the greater increase in cover and height of tall herbaceous plants on the grasslands than at the excavated sites.

Inundation also affected the height of hawthorn. Our results indicate that height was greater on EL than on EM (comparison of the same age classes of low and medium height in Fig. 5). This could be explained by shoot elongation to overcome flooding events. This is demonstrated by Siebel (1998), where partially submerged seedlings of *Q. robur* and *Fraxinus excelsior* L. showed a significantly larger increase in stem length than unflooded ones. Probably, hawthorn uses the same mechanism to survive inundation.

Effects of grazing

On EL, rabbits did not affect the establishment and growth of hawthorn, and the cover and height of grasses and herbaceous plants. Therefore, we conclude that the described effects of herbivores on the establishment and growth of hawthorn were caused by cattle and horses, supporting the third expectation. Bakker et al. (2004) report that the negative effect of rabbits on woody species equals that of cattle. However, in their study area, DEE of rabbits equalled that of cattle, whereas in our study area

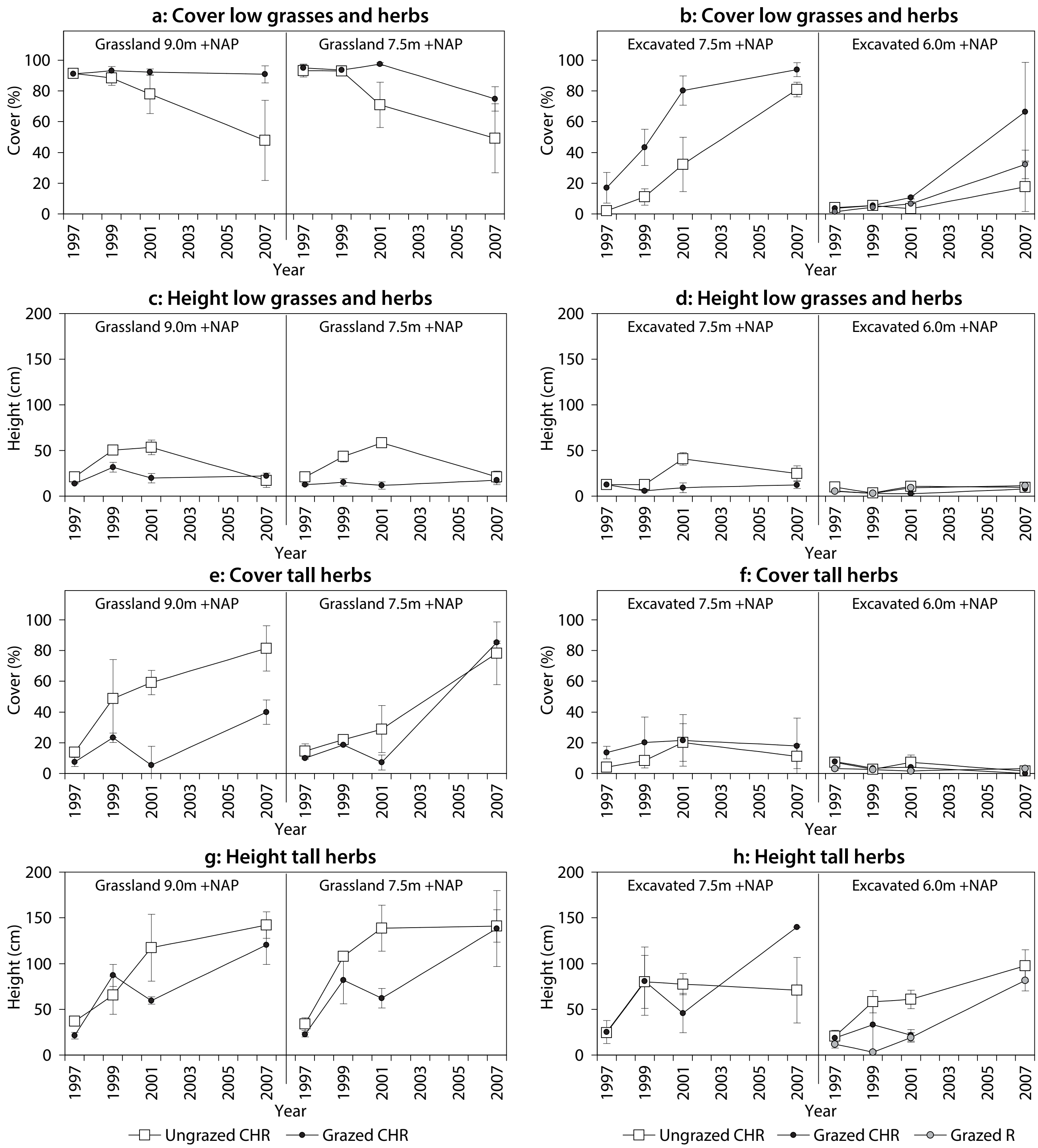

Fig. 6 Development of cover and height of the structural layers "low grasses and herbaceous plants" and "tall herbaceous plants" on different strata and in grazed and ungrazed situations. C = cattle, H = horses, R = rabbits.

DEE of rabbits was >15 times lower than that of cattle and horses during winter. Kuiters and Slim (2003) report significant effects of rabbits on tree regeneration only at very high densities of 50 rabbits per ha, which is about 25 times higher than recorded in our study area.

On the grasslands, there were too few plants of hawthorn to test our fourth expectation. According to the wood-pasture theory (Vera 2000), large herbivores play a key role in the development of park-like landscapes with shrub and tree regeneration in the grazed grasslands. Vera (2000) and Olff et al. (1999) also mention that the intensively grazed grasslands in the woodland-grassland cycle need a temporary reduction in the large herbivore population densities in order to create a "window of opportunity" for the (re-)establishment of shrubs and trees. In our study area, the change in grazing management in 1996 from intensively to extensively grazed, could have been the reason why hawthorn successfully colonized medium high grassland during the first three years of the research period, when cover and height of tall herbaceous plants were still low. In the ungrazed plots after 1998, the cover of tall herbaceous plants had already increased by more than 20% with heights exceeding 1 m. Apparently, this was enough to prevent the establishment of hawthorn and other woody shrubs and trees. In the grazed plots, the cover and height of tall herbaceous plants decreased again in 2001, but this did not result in new establishments of hawthorn, or other woody species, in the grazed plots. This could be due to the fact that within the grazed sites, there were locations where tall herbaceous plant dominated and out competed the hawthorn seedlings for light, whereas at the locations where low grasses and herbaceous plants dominated, local grazing pressure was too high, indicated by the low height of the grasses and herbaceous plants, for the hawthorn seedling to survive. The fact that there were no woody species on GH was probably due to a faster increase in cover and height of the tall herbaceous plant layer and absence of a large input seed carried by water (for example hawthorn) during the first years compared with GM.

Implications for management

This study confirmed that excavation, which increases the discharge capacity of the floodplain, also increased opportunities for shrub and tree regeneration and floodplain forest development. For safety purposes, it is necessary to control the growth of woody species as shrubs and trees can seriously obstruct water flow and reduce the discharge capacity of the river. Our research shows that large herbivores can control the encroachment by woody plants, but much depends on the management of the grazing regime. Although there was a strong negative effect on establishment of hawthorn, the numbers of cattle and horses were insufficient for stopping the development or reducing the growth of this shrub. To suppress hawthorn, more animals and types of herbivores (grazers, intermediate feeders and browsers; large and small herbivores) are needed (e.g. Good et al. 1990; Williams et al. 2010; Cornelissen et al. 2014a).

It is possible to reduce the encroachment of floodplains by woody plants by minimizing their establishment and growth. First of all, it is possible to reduce the areas with ground levels that favour the establishment of shrubs and trees. Another possible measure is to recover excavated areas with the top layer of soil that was removed. This will enhance a rapid recovery of the grass and herbaceous plant layer, diminish the opportunities for the establishment and growth of woody species. A third option is to periodically lower large areas of the floodplain or dig side channels to increase the flow capacity of the floodplain so that a larger area of forest is acceptable. This measure is also known as the "cyclic floodplain rejuvenation" (Baptist et al. 2004). Finally, grazing regimes can be introduced that are more likely to control the development of woody plants. An assemblage of different kinds of herbivores with greater numbers of cattle and horses (>1 animal per ha), more intermediate feeders (e.g. red deer), browsers (e.g. roe deer) and small herbivores (beavers, rabbits and hares) will stimulate browsing. Preferentially, these grazing regimes should start immediately after excavation in order to control establishment for several years, or at least until vegetation cover has developed to a sufficient level.

For rehabilitation of natural floodplain forests, lowering the floodplain and digging side channels are ideal measures for regenerating shrubs and trees in grazed systems. Our research showed that high numbers of hawthorn, a key species in the wood-pasture hypothesis (Vera 2000), can become established and create opportunities for the establishment of palatable and less protected hardwood tree species (Barbosa et al. 2009) and initiate floodplain forest development in these extensively grazed areas.

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REFERENCES

Bakker ES, Olff H, Boekhoff M, Gleichman JM, Berendse F (2004) Impact of herbivores on nitrogen cycling: contrasting effects of small and large species. Oecologia 138: 91–101.

Baptist MJ, Penning WE, Duel H, Smits AJM, Geerling GW, VanderLee GEM, VanAlphen JSL (2004) Assessment of the effects of cyclic floodplain rejuvenation on flood levels and biodiversity along the Rhine river. River Res Appl 20: 285–297.

Baraza E, Zamora R, Hódar A (2006) Conditional outcomes in plant-herbivore interactions: Neighbours matter. Oikos 113: 148–156.

Barbosa P, Hines J, Kaplan I, Martinson H, Szezepaniec A, Szendrei Z (2009) Associational resistance and associational susceptibility: Having right or wrong neighbours. Ann Rev Ecol Evol Syst 40: 1–20.

Bokdam J, Gleichman JM (2000) Effects of grazing by free-ranging cattle on vegetation dynamics in a continental north-west European heathland. J Appl Ecol 37: 415–431.

Brown AG, Harper D, Peterken GF (1997) European floodplain forests: structure, functioning and management. Global Ecol Biogeogr Letters 6: 169–178.

Bunting DP, Papuga S, Grabau M (2013) Long-term vegetation dynamics after high-density seedling establishment: Implications for riparian restoration and management. River Res Appl 29: 1119–1130.

Buttenschøn J, Buttenschøn RM (1985) Grazing experiments with cattle and sheep on nutrient poor, acidic grassland and heath. IV: establishment of woody species. Natura Jutlandica 21: 47–140.

Cornelissen P, VanDeursen EJM, Vulink JT (1995) Year-round grazing at the Zoutkamperplaat: Effects on vegetation and performance of cattle and horses (in Dutch). Ministry of Transport and Public Works. Lelystad, the Netherlands.

Cornelissen P, Gresnigt MC, Vermeulen R, Bokdam J, Smit R (2014a) Transition of a Sambucus nigra L. dominated vegetation into grassland by a multi-species herbivore assemblage. J Nat Conserv 22: 84–92.

Cornelissen P, Bokdam J, Sykora K, Berendse F (2014b) Effects of large herbivores on wood pasture dynamics in a European wetland system. Basic Appl Ecol 15: 396–406.

Decuyper M, Cornelissen P, Sass-Klaassen U (2014) Establishment and growth of hawthorn in floodplains in the Netherlands. Dendrochronologia 32: 173–180.

DeWaal LC, Large ARG, Gippel CJ, Wade PM (1995) River floodplain rehabilitation in Western Europe: opportunities and constraints. Archive für Hydrobiologie 101: 679–693.

Dynesius M, Nilsson C (1994) Fragmentation and flow regulation of river systems in the northern third of the world. Science 266: 753–762.

Garcia D, Chacoff NP (2007) Scale-dependent effects of habitat fragmentation on Hawthorn pollination, frugivory, and seed predation. Conserv Biol 21: 400–411.

Gill R (2006) The influence of large herbivores on tree recruitment and forest dynamics. In: Danell K, Bergström R, Duncan P, Pastor J (eds) Large herbivore ecology, ecosystem dynamics and conservation. Cambridge University Press. Cambridge, UK, pp 170–202.

Good JEG, Bryant R, Carlill P (1990) Distribution, longevity and survival of upland hawthorn (*Crataegus monogyna*) scrub in North Wales in relation to sheep grazing. J Appl Ecol 27: 272–283.

Herrera CM (1984) Seed dispersal and fitness determinants in wild roses. Combined effects of hawthorn, birds, mice, and browsing ungulates. Oecologia 63: 386–393.

Hester AJ, Bergman M, Iason GR, Moen J (2006) Impacts of large herbivores on plant community structure and dynamics. In: Danell K, Bergström R, Duncan P, Pastor J (eds) Large herbivore ecology, ecosystem dynamics and conservation. Cambridge University Press. Cambridge, UK, pp 97–141.

Hughes FMR, Adams WM, Muller E, Nilsson C, Richards KS, Barsoum N, Decamps H, Foussadier R, Girel J, Guilloy H, Hayes A, Johansson M, Lambs L, Pautou G, Peiry J-L, Perrow M, Vautier F, Winfield M (2001) The importance of different scale processes for the restoration of floodplain woodlands. Regulated Rivers: Res Manag 17: 325–345.

Hurlbert SH (1984) Pseudoreplication and the design of ecological field experiments. Ecol Monogr 54: 187–211.

Jones RH, Sharitz RR, McLeod KW (1989) Effects of flooding and root competition on growth of shaded bottom land hard wood seedlings. Am Midl Nat 121: 165–175.

Kuiters AT, Hazebroek E, Hennekens M (2003) Actualized overview of grazed nature conservation areas in the Netherlands (in Dutch). Natuurbeheer 6: 79–82.

Kuiters AT, Slim PA (2003) Tree colonization of abandoned arable land after 27 years of horse-grazing: the role of bramble as a facilitator of oak wood regeneration. For Ecol Manag 181: 239–251.

Lange R, Twisk P, VanWinden A, VanDiepenbeek A (1994) Mammals of Western Europe (in Dutch). KNNV. Utrecht, the Netherlands.

Leyer I, Mosner E, Lehmann B (2012) Managing floodplain-forest restoration in European river landscapes combining ecological and flood-protection issues. Ecol Appl 22: 240–249.

Makaske B, Maas GJ, VandenBrink C, Wolfert HP (2011) The influence of floodplain vegetation on hydraulic roughness: is ecosystem rehabilitation in Dutch embanked floodplains compatible with flood safety standards? Ambio 40: 370–376.

Martinez I, Garcia D, Obeso JR (2008) Differential seed dispersal patterns generated by a common assemblage of vertebrate frugivores in three fleshy-fruited trees. Ecoscience 15: 189–199.

Naiman RJ, Décamps H (1997) The ecology of interfaces: The riparian zone. Ann Rev Ecol Syst 28: 621–658.

Naiman RJ, Bunn SE, Nilsson C, Petts GE, Pinay G, Thompson LC (2002) Legitimizing fluvial ecosystems as users of water. Environ Manag 30: 455–467.

Niinemets U, Valladares F (2006) Tolerance to shade, drought, and water logging of temperate Northern Hemisphere trees and shrubs. Ecol Monogr 76: 521–547.

Norusius MJ (2006) SPSS for windows. SPSS Inc. Chicago, USA.

Olff H, Vera FWM, Bokdam J, Bakker ES, Gleichman JM, de Maeyer K, Smit R (1999) Shifting mosaics in grazed woodlands driven by the alternation of plant facilitation and competition. Plant Biol 1: 127–137.

Olson DM, Dinerstein E (1998) The global 200: a representation approach to conserving the earth's most biologically valuable ecosystems. Conserv Biol 12: 501–512.

Rosenberg DM, McCully P, Pringle CM (2000) Global-scale environmental effects of hydrological alterations: Introduction. Bioscience 50: 746–751.

Siebel HN (1998) Floodplain forest restoration. Tree seedling establishment and tall herb interference in relation to flooding and shading. PhD Thesis, Nijmegen University the Netherlands.

Smith CJ (1980) Ecology of the English chalk. Academic Press. London, UK.

Snow B, Snow D (1988) Birds and Berries. A study of an ecological interaction. A&C Black Publishers Ltd. London, UK.

Sokal RR, Rohlf FJ (1981) Biometry. The principles and practice of statistics in biological research (2nd ed.). W.H. Freeman and Company. New York, USA.

Streng DR, Glitzerstein JS, Hardcombe PA (1989) Woody seedling dynamics in an East Texas Floodplain forest. Ecol Monogr 59: 177–204.

Tockner K, Stanford JA (2002) Riverine flood plains: present state and future trends. Environ Conserv 29: 308–330.

Van der Meijden R (2005) Flora of the Netherlands (23rd ed.; in Dutch). Wolters-Noordhof. Groningen, the Netherlands.

Van Splunder I (1998) Floodplain forest recovery. Softwood forest development in relation to hydrology, riverbank morphology and management. PhD Thesis, Nijmegen University the Netherlands.

Van Velzen EH, Jesse P, Cornelissen P, Coops H (2003) Water resistance of vegetation in floodplains (in Dutch). Ministry of Transport and Public Works. RIZA report nr. 2003.028. Lelystad, the Netherlands.

Vera FWM (2000) Grazing ecology and forest history. CAB International. Wallingford, UK.

Vreugdenhil SJ, Kramer K, Pelsma T (2006) Effects of flooding duration, frequency and depth on the presence of saplings of six woody species in north-west Europe. For Ecol Manag 236: 47–55.

Watt AS (1934) The vegetation of the Chiltern Hills with special reference to the beechwoods and their seral relationships. Part II. J Ecol 22: 445–507.

Wallage-Drees JM (1988) Rabbits in the coastal sand dunes; weighed and counted. PhD Thesis, Leiden University the Netherlands.

Williams PA, Kean JM, Buxton RP (2010) Multiple factors determine the rate of increase of an invading non-native tree in New Zealand. Biol Invasions 12: 1377–1388.

STUDY OF THE EFFECTS OF MYCORRHIZA, FULVIC ACID, SEAWEED EXTRACT AND UREA ON PHYSIOLOGICAL TRAITS AND LEAF YIELD OF TOBACCO (BURLEY 21)

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ABSTRACT

To study the effects of mycorrhiza, fulvic acid, seaweed extract and urea on the physiological traits and leaf yield of tobacco, an experiment was carried out in split plots in a randomized complete block design with three replicates at Marivan during 2015. In this study, the main factor was two levels of inoculation with mycorrhiza including: control: no inoculation and inoculation with mycorrhiza (*Glomus interaradices*) and subplots treated with growth stimulants at 5 kinds: control-distilled water, fulvic acid, seaweed extract, urea, fulvic acid + seaweed extract + urea. The results indicate that the number of leaves and dry weight of the middle leaves of plants inoculated with mycorrhiza were increased significantly. In addition, the foliar application of growth stimulants significantly affected the relative water content, fresh and dry weight of the lower and middle leaves and also the greenness and fresh weight of the upper leaves of tobacco.

Keywords: canopy; dry weight; fertilizer; growth promotion; symbiosis

Introduction

Although the application of chemical fertilizers has resulted in a huge increase in world food production (Banerjee et al. 2011; Garai et al. 2014), it has contributed significantly to the pollution of water, air and soil (Ignacimuthu and Vendan 2007). Phosphorus has been applied regularly for many years and the level of P is now relatively high at some locations (Magarey et al. 2005). Tobacco is a cash crop and the best quality tobacco comes from crops cultivated in regions in Iran where it is humid and the soil is fertile. However, it is important that tobacco does not contain toxic or heavy metals. To achieve this it is vital to reduce the use of chemical fertilizers that contain toxic and heavy elements, such as cadmium, because these fertilizers increase the risk of Cd entering the food chain, which has an adverse effect on the kidneys, lungs, cardiovascular and musculoskeletal systems of humans (Roberts 2014). In order to prevent or decrease the incidence of such problems it is necessary to produce healthy crops by using environmentally friendly fertilizers such as organic fertilizers or biofertilizers instead of chemical fertilizers. Arbuscular mycorrhiza is known to assist plants in their uptake of phosphorus and certain trace elements such as zinc and copper. The mycorrhiza invades the root system of its host and utilizes carbon from the plant and in return effectively increasing the soil volume from which the plant may draw nutrients. This enables plants to produce high yields even when growing in soil with low levels of P (Magarey et al. 2005). Increasing concentrations of N, P, K and S in the rhizosphere and dry weight of onion bulbs and maize shoots by inoculating these plants with mycorrhizae is reported (Amal et al. 2014). Munda et al. (2016) report better productivity of soybean when grown in plots treated with biofertilizers and that the microbes also improve P availability for the next crop.

Fulvic acid is an organic fertilizer with a non-toxic mineral chelating additive and water binder that maximizes its uptake through leaves and stimulates plant productivity (Malan 2015). It attracts water molecules, which helps in keeping soil moist and aids the movement of nutrients into plant roots. Fulvic acid easily binds or chelates minerals such as iron, calcium, copper, zinc and magnesium, and can deliver these elements directly to plants (Yamauchi et al. 1984). Various studies report that it increases the fluorescence of chlorophyll a, inhibits ROS and enhances the antioxidant enzymes that destroy ROS (Lotfi et al. 2015), decreases water stress or stress due to hot and dry winds during ear development, increasing grain yield by 7.3–18.0%, enhances root activity, increases ion uptake and the rate of transport of phosphorus to the grains (Xudan 1987), increases the number and length of root hairs in Arabidopsis plants (Schmidt et al. 2005), ameliorates the growth of rice and radish resulting in taller plants (Khang 2011), limits the development of some pathogens, e.g. Fusarium spp (Yigit and Dikilitaş 2008) and the availability of phosphorus (Yang et al. 2013). Seaweed extract, another organic fertilizer, contains micro and macro nutrients and growth promoters (Prasad et al. 2010). It also contains plant growth hormones that enhance yield (Latique et al. 2013). Application of seaweed extract stimulates growth and yield and increases the tolerance of plants to environmental stress (Pramanick et al. 2013), improves germination of maize seeds by 10–19%, promotes shoot and root growth by 30–68% (Matysiak et al. 2011), increases the resistance of herbaceous plants to drought (Zhang and Ervin 2004), promotes vegetative growth, yield and oil content of peanuts (31.69%, 14.27%) and sunflower (31.69%, 14.27%),

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respectively (Karthikeyan and Shanmugam 2015). In addition, spraying plants with seaweed extract improves growth, yield and tuber quality of potatoes in terms of their nitrogen, total soluble solids and protein contents (Haider et al. 2012), increases root growth, nutrient absorption, stem thickness and growth (Jensen 2004), ameliorates effect of salt stress on bean seeds because of the presence of growth hormones, nutrients and other important phytochemical compounds (Latique et al. 2014).

Therefore, this study compares the effectiveness of organic and chemical fertilizers in terms of the increase in yield of leaf tobacco.

Material and Methods

This experiment was carried out in split-plots based on a randomized complete block design with three replicates in a farmer's tobacco field at Marivan in the northwest of Iran (situated at 35°N and 46°E and at 1324 m above mean sea level), where the annual precipitation during the 2015 growing season was 750 mm. In this study, the main factor was the inoculation of the mycorrhizal fungus (*G. intaradices*) and a control (not inoculated) and the sub-factor application of growth compounds of 6 kinds: distilled water (control), fulvic acid, seaweed extract, urea, fulvic acid + seaweed extract + urea). The fulvic acid was prepared by Fanavari Sabz Shargh Co, Tehran, Iran. The seaweed extract (Acadian™) was made from *Ascophyllum nodosum* obtained from Nova Scotia, Canada. Sterilized and coated seeds of tobacco (*Nicotiana tabacum* cv. Burley 21) were obtained from the Iranian Tobacco Administration. These seeds were germinated in a float tray in a greenhouse two months before planting in a field. At the end of May, seedlings were transplanted to another field and planted 40 cm apart in rows that were 1 meter apart. For the inoculation of mycorrhiza, mycorrhiza powder (3.33 g/p) was placed in rows and then covered with 1 cm of soil prior to transplanting the seedlings. The growth compound were sprayed (2/1000 w/v concentration) on the plants one month before the lower leaves were harvested. The watering of the plants was achieved using furrows. At various stages of the growth of the tobacco plants they were watered and weeds were removed. In this study, the following characters were recorded after spraying: the greenness, temperature and relative water content of the leaves. The greenness was based on the readings from 10 leaves (lower, middle, upper) using a Chlorophyll Meter SPAD-502 and the temperature of the same leaves using an Infra-red Thermo meter CEMDT-8810. Relative water content was measured using Baslam and Goicoechea (2012) method.

$$RWC = 100 \times (FW - DM) / (TM - DM)$$

In this formula, FW is fresh weight, DM dry weight and TW turgid weight.

Fresh weight is that of ten 2 cm diameter pieces of leaves that were previously kept in an ice box. In order to calculate TM, the leaf pieces were placed in distilled water for 24 h, wiped to remove excess water and then weighed. DM was obtained by drying the leaf pieces in an oven at 75 °C for 48 h before weighing them. At harvest, the lower, middle and upper leaves were collected from two plants in each plot together with a record of the number and dry and fresh weight of the leaves. Finally, data were analyzed according to the experimental design in $SAS_{9.1}$ software package and comparison of the trait means using Duncan's multiple range test at 0.05 probability.

Results and discussion

Leaf temperature

Leaf temperature was not affected significantly by the mycorrhiza inoculations (Table 1), but as shown in Table 2, they resulted in a decrease in the temperature of the middle and upper leaves by improving the water balance of the plants, possibly by increasing the uptake of water from the soil. Probably, if the plants had been subject to water stress then a greater effect of mycorrhiza would have been recorded as Bakr et al. (2018) report that inoculation of mycorrhiza decreases canopy temperature significantly only in water-limited conditions. Also Chen et al. (2017) conclude that mycorrhiza enhances the gas exchange capacity of plants by improving "stomatal opening, reducing stomatal resistances and increasing transpiration fluxes". The effects of mycorrhiza in decreasing the temperature of leaves is due to their increasing water absorption, improving water use efficiency and effect on stomatal conductance (Chen et al. 2017; Bakr et al. 2018). Probably the high rainfall and air humidity in the area studied accounts for the non-significant effect of mycorrhiza on leaf temperature.

Also, the effects of foliar application of growth compounds and interaction between inoculation with mycorrhiza and foliar application were not significantly different. As shown in Table 2, despite the no significant difference between treatments, the foliar application of growth compounds decreased canopy temperature compared with the control. The minimum temperature of the middle leaves was recorded when fulvic acid was applied. Xudan (1987) report that foliar application of fulvic acid improves resistance to drought in wheat via partial closure of the stomata, which reduces transpiration and raises the water potential during flowering; it also enhances root activity. In this study, a significant effect of growth compound was not recorded as the field was irrigated and the rainfall and humidity were high.

Greenness

This character was not affected significantly by inoculation with mycorrhiza as it increased slightly in the lower and upper but not in middle leaves. It is concluded that the increase in availability of nutrients, especially nitrogen, due to inoculation with mycorrhiza (cf. Amal et al. 2014) can affect greenness, as nitrogen is needed for producing chlorophyll. Chen et al. (2017) also reports that the inoculation with mycorrhiza increases the contents of some nutrients such as nitrogen and therefore of chlorophyll. Bakr et al. (2018) also report that inoculation with mycorrhiza enhances the relative chlorophyll index only under drought conditions with no increase when there is an optimum supply of water.

In this study, the foliar application of growth compounds had a significantly different effect on the greenness of the upper leaves (Table 1). As shown in Table 2 it had a positive effect on the greenness of the lower, middle and upper leaves compared with the control. However, for the upper leaves the maximum greenness was recorded for those treated with seaweed extract, fulvic acid and urea. It is possible that the nutrients in the soil, especially nitrogen, are sufficient to meet the needs of the plants until the middle of the vegetative season, but after that when there was a reduced level of nutrients in the soil the application of growth compounds resulted in an increase in chlorophyll in the upper leaves. This result may also be attributed to the location and exposure of the upper leaves to sunlight along with the translocation of nitrogen to the upper parts of the plant, especially the upper leaves.

These results are in accordance with the findings of other researchers. Xudan (1987) reports that spraying with fulvic acid resulted in a higher level of chlorophyll in the leaves and a greater uptake by the roots in wheat and Lotfi et al. (2015) report that the application of fulvic acid improved the maximum quantum efficiency of PSII (Fv/Fm) and the performance index (PI) of plants under both well-watered and drought conditions. They also report that application of FA significantly increases the fluorescence of chlorophyll a, inhibits ROS production and enhances antioxidant enzymes activity that destroys ROS.

Relative water content

Relative water content was not affected significantly by inoculation with mycorrhiza, but there was an increase in relative water content of all the leaves (Table 2). It is likely that mycorrhiza could increase relative water content via increasing root growth and the uptake of phosphorus from soil (Magarey et al. 2005), resulting in greater water uptake and increase in the RWC of the leaves. As it was mentioned in the results of leaf temperature, if this experiment was conducted under limited irrigation or rainfed conditions, increasing water absorption and consequently, enhancing relative water content of the leaves likely to be done by mycorrhiza.

In this study, the foliar application of growth compounds on the RWC in the lower leaves differed signif-

Table 1 Results of the analysis of variation of the effects of mycorrhiza, fulvic acid, seaweed extract and urea on the physiological traits of tobacco.

SOV	df	Leaf temperature			Greenness			RWC		
		Lower	Middle	Upper	Lower	Middle	Upper	Lower	Middle	Upper
Block	2	0.99^{ns}	10.59^{ns}	12.85^{ns}	12.42^{ns}	36.75^{ns}	7.46^{ns}	52.93^{ns}	442.75**	19.89^{ns}
Mycorrhiza	1	1.79^{ns}	2.91^{ns}	7.29^{ns}	3.52^{ns}	24.24^{ns}	10.87^{ns}	102.52^{ns}	15.19^{ns}	145.99^{ns}
Ea	2	5.58	5.99	10.89	5.45	44.60	1.32	259.94	6.40	378.86
Growth compounds	4	1.87^{ns}	4.04^{ns}	0.88^{ns}	3.68^{ns}	17.09^{ns}	16.66*	145.33*	34.37^{ns}	58.52^{ns}
M × G	4	1.10^{ns}	2.73^{ns}	1.33^{ns}	2.90^{ns}	11.58^{ns}	1.47^{ns}	32.98^{ns}	80.08^{ns}	80.01^{ns}
Eb	16	2.06	5.53	2.77	2.60	9.38	7.24	50.24	61.82	23.93
CV (%)		5.27	8.56	5.64	4.48	9.48	7.30	10.83	10.66	16.06

**, * Significant at 1% and 5% level, respectively, ^{ns} non-significant.

Table 2 Comparison of the average effects of mycorrhiza, fulvic acid, seaweed extract and urea on the physiological traits of tobacco.

Treatment		Leaf temperature (°C)			Greenness			RWC (%)		
		Lower	Middle	Upper	Lower	Middle	Upper	Lower	Middle	Upper
Mycorrhiza	Control	26.984^{a}	27.781^{a}	30.027^{a}	35.958^{a}	33.189^{a}	36.224^{a}	63.562^{a}	72.987^{a}	67.015^{a}
	Inoculation	27.473^{a}	27.158^{a}	29.041^{a}	36.280^{a}	31.391^{a}	37.482^{a}	67.259^{a}	74.410^{a}	71.521^{a}
Growth compounds	Control	28.320^{a}	28.565^{a}	29.886^{a}	35.228^{a}	30.875^{a}	34.010^{b}	63.182^{b}	71.280^{a}	70.342^{a}
	Seaweed	27.850^{a}	27.192^{a}	29.206^{a}	36.251^{a}	30.975^{a}	37.815^{a}	64.000^{b}	73.090^{a}	67.897^{a}
	Fulvic	27.665^{a}	21.318^{a}	30.003^{a}	37.186^{a}	31.678^{a}	37.742^{a}	65.147^{ab}	72.088^{a}	67.698^{a}
	Urea	26.686^{a}	27.770^{a}	29.193^{a}	35.551^{a}	33.035^{a}	37.970^{a}	60.950^{b}	74.722^{a}	66.277^{a}
	Seaweed+fulvic+urea	26.621^{a}	27.503^{a}	29.381^{a}	35.525^{a}	34.885^{a}	36.593^{ab}	73.775^{a}	77.312^{a}	74.225^{a}

Averages with the same letter in each column are not significantly different at a 0.05 probability level using Duncan's multiple range test.

icantly (Table 1). The maximum RWCs were recorded in the lower leaves when fulvic acid + seaweed extract + urea was applied. It also had a positive effect on the RWCs of the middle and upper leaves, but it was not significant. It is concluded that the higher level of RWC in lower leaves may be due to their proximity to the roots and receiving a larger amount of growth compound due to their larger size when the growth compounds were applied. This is supported by the decrease in the RWC of the middle and a greater decrease in that of the upper leaves of the plant (Table 2). On the other hand, the middle and upper leaves are more exposed to sunlight and consequently transpire more water, therefore did not show a significant response to this compound.

Xudan (1987) reports that the foliar application of fulvic acid improves the drought tolerance of wheat by inducing a partial closure of the stomata, which reduces transpiration and increases the water potential during flowering and enhances root activity, resulting in an increase in ion uptake and high rate of transport of phosphorus to the grains.

Numbers of leaves

Inoculation with mycorrhiza significantly affects the number of middle leaves on tobacco plants (Table 3). Comparison of the average number of middle leaves indicates they are more numerous on plants inoculated with mycorrhiza. Number of leaves is very important, in term of leaf yield and consequently farmer income. All tobacco plants produce about 20–50 leaves in a growing season. In addition, most of the highest quality leaves are middle leaves, which have lower nicotine content.

As it takes time for the colonization and growth of fungal mycelium and the onset of symbiosis and as a consequence the positive effect of this symbiosis is only likely to affect the middle and upper leaves and then increases in air and soil temperature reduce its effect. As shown by Augé et al. (2015) mycorrhiza promotes an increase of about 10% when air temperature is less than 27 °C but negative increases at higher temperatures. In another study, it is reported that an increase in air temperatures of 3 °C reduces colonization by mycorrhiza (Wilson et al. 2016). The decline in the activity of mycorrhiza at high temperatures is also reported by Mohan et al. (2014).

However, increased leaf numbers, leaf area, shoot and root biomass and higher chlorophyll a, b in plants inoculated with mycorrhiza is also reported (Lu and Wu 2017).

There were no significant effects of foliar applications of growth compounds on the number of leaves (Table 3). Comparison of the average numbers of leaves reveal that the highest number of both lower and middle leaves developed on plants sprayed with seaweed extract and fulvic acid + seaweed extract + urea. Latique et al. (2013)

Table 3 Results of the analysis of variance of the effects of mycorrhiza, fulvic acid, seaweed extract and urea on the leaf traits of tobacco.

SOV	df	Numbers of leaves			Leaf fresh weight			Leaf dry weight		
		Lower	Middle	Upper	Lower	Middle	Upper	Lower	Middle	Upper
Block	2	0.433^{ns}	7.05**	2.508^{ns}	0.0001^{ns}	0.003^{ns}	0.0004^{ns}	9.33^{ns}	0.00006^{ns}	0.000008^{ns}
Mycorrhiza	1	0.133^{ns}	35.20**	0.008^{ns}	0.0003^{ns}	0.530^{ns}	0.00002^{ns}	8.53^{ns}	0.0002*	0.00004^{ns}
Ea	2	1.033	0.108	1.108	0.0010	0.378	0.0004	0.00002	0.000009	0.00003
Growth compounds	4	0.395^{ns}	1.80^{ns}	0.32^{ns}	0.0010**	0.063**	0.0068**	0.00003*	0.0008**	0.00007^{ns}
M × G	4	0.112^{ns}	0.18^{ns}	0.195^{ns}	0.0002^{ns}	0.009^{ns}	0.0005^{ns}	0.00001^{ns}	0.00005^{ns}	0.00006^{ns}
Eb	16	0.191	5.55	1.277	0.0002	0.012	0.0013	0.00001	0.0001	0.00008
CV (%)		10.100	14.09	19.880	11.21	15.82	14.35	16.7	7.94	19.11

**, * Significant at 1% and 5% levels, respectively, ^{ns} non-significant.

Table 4 Comparison of the average effects of mycorrhiza, fulvic acid, seaweed extract and urea on the leaf traits of tobacco.

Treatment		Numbers of leaves			Leaf fresh weight (g/p)			Leaf dry weight (g/p)		
		Lower	Middle	Upper	Lower	Middle	Upper	Lower	Middle	Upper
Mycorrhiza	Control	4.266^{a}	15.630^{b}	5.700^{a}	0.148^{a}	0.677^{a}	0.225^{a}	0.020^{a}	0.127^{b}	0.048^{a}
	Inoculation	4.400^{a}	17.800^{a}	5.660^{a}	0.154^{a}	0.752^{a}	0.253^{a}	0.021^{a}	0.133^{a}	0.046^{a}
Growth compounds	Control	4.000^{a}	16.583^{a}	5.916^{a}	0.130^{b}	0.598^{c}	0.252^{abc}	0.019^{ab}	0.143^{a}	0.052^{a}
	Seaweed	4.583^{a}	17.250^{a}	5.500^{a}	0.166^{a}	0.834^{a}	0.241^{bc}	0.023^{a}	0.130^{ab}	0.049^{a}
	Fulvic	4.166^{a}	16.917^{a}	5.916^{a}	0.158^{ab}	0.762^{a}	0.290^{a}	0.022^{a}	0.140^{a}	0.047^{a}
	Urea	4.333^{a}	15.833^{a}	5.666^{a}	0.132^{b}	0.609^{bc}	0.205^{c}	0.017^{b}	0.114^{c}	0.045^{a}
	Seaweed+fulvic+urea	4.583^{a}	17.000^{a}	5.416^{a}	0.163^{a}	0.745^{ab}	0.281^{ab}	0.022^{a}	0.124^{bc}	0.043^{a}

Averages with the same letter in each column are not significantly different at a 0.05 probability level using Duncan's multiple range test.

report that seaweed contains growth hormones that enhance yield. The same results are reported for potato (Sarhan 2011; Haider et al. 2012), peanut and sunflower (Karthikeyan and Shanmungam 2015). Conversely, when the initial soil organic matter and NO3-N levels are high seaweed extract has no effect on yield (Wickham and Davis 2015).

Since, in this experiment the leaves developed before spraying, the fact they did not increase in number is reasonable, but the size or weight of the leaves were expected to increase.

Leaf fresh weight

Leaf fresh weight was not affected significantly by inoculation with mycorrhiza (Table 4). Like the effects of mycorrhiza on RWC, this is to be expected, because mycorrhiza helps plants to obtain water, however, the effect was not significantly different.

Bakr et al. (2018) report that fresh weight of moderately stressed plants inoculated with mycorrhiza was increased by 73% but for those well supplied with water it was only 8%.

The effect of foliar applications of growth compounds on leaf fresh weight were significantly different for all leaves. The maximum leaf fresh weight was recorded for the lower leaves of plants sprayed with seaweed extract and fulvic acid + seaweed extract + urea and for the middle leaves sprayed with seaweed extract and fulvic acid and for upper leaves with fulvic acid. It seems that, the difference in the effect of growth compound is due to the variation in the needs of tobacco leaves in different positions, due to their age, size, exposure to solar radiation and temperature.

Esringu et al. (2015) show that humic and fulvic acids promote plant growth in terms of the number of buds, plant height, number of main shoots, number of side shoots, plant diameter, root length, fresh root weight and fresh shoot weight.

Leaf dry weight

Inoculation with mycorrhiza had a significant effect on the dry weight of the middle leaves (Table 4). Probably this is due to an increase in the absorption of macro and micronutrients from the soil as is reported by Amal et al. (2014) and Munda et al. (2016).

Chen et al. (2017) report that the net photosynthetic rate and stomatal conductance is increased significantly in plants inoculated with mycorrhiza by 58.76 and 95.65%, respectively. However, for other species of mycorrhiza, the increase in these traits is reported to be 35.79 and 21.26%. They conclude that the enhancement of photosynthesis in these treatments is associated with an increase in ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) and Calvin cycle enzymes. Finally, they state that plant height, dry weight of shoot and root, root to shoot ratio, root activity, chlorophyll content, photosynthetic characteristics and nutrient uptake, including N, P, K, Ca, Cu, Fe, Mn, Mg, Zn, and S of inoculated plants are all significantly higher. In another study, the improvement in the growth of plants inoculated with mycorrhiza is due to improvements in water use efficiency, stomatal conductance and photosynthetic efficiency (Bakr et al. 2018).

Mycorrhizal symbiosis can greatly improve yield when the level of P in the soil is low (Magarey et al. 2005), hence we conclude that if pre-sowing application of chemical fertilizers had not occurred in this experiment, it is likely the yield increase would have been higher. The results of Munda et al. (2016) indicate that the application of biofertilizers can improve the availability of P for the next crop. However, Hendrix (1993) indicates that the symbiosis between mycorrhiza and tobacco reduces the yield of commercial tobacco and is the cause of a poor growth condition called "tobacco stunt".

In this study, the foliar application of growth compounds resulted in significantly different dry weights of the lower and middle leaves. The maximum weight of lower leaves was recorded for the plants sprayed with seaweed extract, fulvic acid and fulvic acid + seaweed extract + urea. For the middle leaves it was for plants sprayed with distilled water and fulvic acid.

The lack of an effect of the growth compounds in increasing the dry weight of the middle and upper leaves can be attributed to two factors. Initially, these growth stimulants may have increased leaf quality, such as their colour, size, shape and soluble sugar content, which were not measured in this experiment. Secondly, since the inflorescence is cut off when the upper leaf of tobacco ceases growing (known as: Topping), thus removing a strong drain on photosynthetic products, which enables these products to be transferred to the growing leaves and therefore obscuring any effects the growth compounds may have had. The increase in the photosynthesis of the leaves may also be similarly determined.

Wickham and Davis (2015) also report that the application of seaweed extract does result in an increase in yield when the soil initially contains high levels of organic matter and NO3-N. The positive results of growth compounds are also reported by others such as: grain yield increase of 7.3–18.0% with application of fulvic acid (Xudan 1987), promoting effects of fulvic acid on dry weights of roots and shoots (Esringu et al. 2015), enhancing of antioxidant enzymes by fulvic acid (Lotfi et al. 2015), significant increase in plant height, number branches and the percentage dry matter in shoots of pepper after application of seaweed extracts (Marhoon and Abbas 2015) and increase in root growth, nutrient element absorption and stem thickness and growth after application of seaweed extracts (Jensen 2004). Sutharsan et al. (2014) also state that the foliar application of seaweed extract increases shoot dry weight (80.92%), root dry weight (81.57%), fruit number (57.87%), fruit yield per hectare (58.70%), Total Soluble Solids (25.71%) and Total acidity (76.95%) content of fruit over that in the control.

Conclusion

Tobacco is a cash crop grown in the northwest of Iran-Marivan and in order to increase the yield of leaves it is planted in fertile soil and treated with commercial fertilizers. This is likely to result in the entry of chemical and heavy elements in the human food chain and in damaging the environment. The application of bio-growth promoters was introduced as an environmentally friendly alternative to chemical fertilizers. In order to increase the yield of tobacco leaves, in this experiment a mycorrhizal fungus (*G. intaradices*) and the bio-stimulants fulvic acid, seaweed extract and chemical stimulant urea were used.

The results of this study indicate that the inoculation of mycorrhiza had positive effects on some of the characters studied, such as an increase in the number and dry weight of the middle leaves of tobacco. Inoculation with mycorrhiza increased the number and dry weight of the leaves by 13.88 and 4.72%, respectively, in comparison with the control. Since the number and weight of leaves determines the final yield and income of the farmers, increasing these components is very desirable. There were positive effects of mycorrhiza on other traits but they were not significant. It is concluded that due to irrigation and high rainfall in the area studied, the positive effects of mycorrhiza was less apparent than expected. In addition, soil fertility and the application of chemical fertilizers before planting may have reduced the positive effects of mycorrhiza. Lower colonization of plants by mycorrhiza is reported when the levels of potassium and magnesium in the soil are high (Zare-Maivan et al. 2017).

On the other hand, mycorrhiza has a more positive effect on plants subject to drought and high salinity. Fernández-Lizarazo and Moreno-Fonseca (2016) conclude that mycorrhiza can enhance the water potential, stomatal conductance and nutritional content of plants and finally result in an increase in plant growth during periods of drought. Also a higher dry mass and leaf area and enhancement of chlorophyll content, light energy absorption, gas exchange and Rubisco activity of plants inoculated with mycorrhiza growing under salinity stress is reported by Xu et al. (2018).

Corrêa et al. (2006) report that the response of plants to mycorrhiza depends on "their age (stage of development, leaf area), their initial nutritional status and the amount of nitrogen supplied". They conclude that colonization by mycorrhiza only increases the net photosynthesis rate in young plants and the application of high levels nitrogen inhibits the formation of mycorrhizal symbioses.

In this experiment, probably the effects of the mycorrhizal fungi was less that of the native strains in the soil, because the soil in the pots was sterilized, which is not possible to duplicate under field condition. In other research, higher dry and fresh weights and chlorophyll contents are reported in plants with mycorrhiza than in those grown in sterilized soil (Zare-Maivan et al. 2017).

Gazey et al. (2004) report that indigenous mycorrhizal fungi colonized a higher proportion of roots in two agriculture soils than introduced species. Hepper et al. (1998) compared three species of mycorrhizal fungus and confirm the superiority of indigenous over introduced species.

Williams et al. (2012), based on studies on six species of mycorrhiza, confirms the superiority of indigenous strains and state that it is better to use indigenous than commercial strains, both in terms of increased plant growth and cost, when regenerating forest.

Also based on the results of this study, the highest numbers of leaves were produced by seaweed and fulvic acid + seaweed extract + urea in the lower leaves side. In lower leaves, the maximum leaf dry weight was obtained from seaweed, fulvic acid and fulvic acid + seaweed extract + urea.

Fulvic acid and seaweed extract contain different macro and micronutrients that can enhance the growth and development of plants resulting in a better quality and greater yield. Matysiak et al. (2011) report that, the spraying of plants with seaweed extract and fulvic acid increased chlorophyll levels by 27–30% and shoot and root weight by 30–68%.

As most of the leaves used to produce tobacco are middle leaves, inoculating tobacco plants with mycorrhiza and the foliar application of growth compounds, especially seaweed extract, can be used to significantly increase the quantity and quality of these leaves.

REFERENCES

Amal AM, Wedad E, Eweda E, Heggo AM, Enas AH (2014) Effect of dual inoculation with arbuscular mycorrhizal fungi and sulphur-oxidising bacteria on onion (*Allium cepa* L.) and maize (*Zea mays* L.) grown in sandy soil under greenhouse conditions. Ann Agr Sci 59: 109–118, http://dx.doi.org/10.1016/j.aoas.2014.06.015.

Augé RM, Toler HD, Saxton AM (2015) Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a metaanalysis. Mycorrhiza 25: 13–24, http://dx.doi.org/10.1007/s00572-014-0585-4.

Bakr J, Pék Z, Helyes L, Posta K (2018) Mycorrhizal inoculation alleviates water deficit impact on field-grown processing tomato. Pol J Environ Stud 27: 1949–1958, https://doi.org/10.15244/pjoes/78624.

Banerjee A, Datta JK, Mondal NK, Chanda T (2011) Influence of integrated nutrient management on soil properties of old alluvial soil under mustard cropping system. Commun Soil Sci Plan 42: 2473–2492, http://dx.doi.org/10.1080/03650340.2014.1000877.

Baslam M, Goicoechea N (2012) Water deficit improved the capacity of Arbuscular Mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. Mycorrhiza 22: 347–359, http://dx.doi.org/10.1007/s00572-011-0408-9.

Chen S, Zhao H, Zou C, Li Y, Chen Y, Wang Z, Jiang Y, Liu A, Zhao P, Wang M, Ahammed GJ (2017) Combined inoculation with multiple Arbuscular mycorrhizal fungi improves growth, nutrient uptake and photosynthesis in cucumber seedlings. Front Microbiol 8: 1–11, http://dx.doi.org/10.3389/fmicb.2017.02516.

Corrêa A, Strasser RJ, Martins-Loução MA (2006) Are Mycorrhiza Always Beneficial? Plant Soil 279: 65–73, https://doi.org/10.1007/s11104-005-7460-1.

Esringü A, Sezen I, Aytatli BA, Ercişli S (2015) Effect of humic and fulvic acid application on growth parameters in *Impatiens walleriana* L. Akademik Ziraat Dergisi 4: 37–42, http://azd.odu.edu.tr/files/4_1/37–42.pdf.

Fernández-Lizarazo JC, Moreno-Fonseca LP (2016) Mechanisms for tolerance to water-deficit stress in plants inoculated with Arbuscular mycorrhizal fungi. A review. Agron Colomb 34: 179–189.

Garai TK, Datta JK, Mondal NK (2014) Evaluation of integrated nutrient management on Boron rice in alluvial soil and its impacts upon growth, yield attributes yield and soil nutrient status. Arch Agron Soil Sci 60: 1–14, http://dx.doi.org/10.1080/03650340.2013.766721.

Gazey C, Abbott LK, Robson AD (2004) Indigenous and introduced Arbuscular mycorrhizal fungi contribute to plant growth in two agricultural soils from south-western Australia. Mycorrhiza 14: 355–362, http://dx.doi.org/10.1007/s00572-003-0282-1.

Haider MW, Ayyub CM, Pervez MA, Asad HU, Manan A, Raza SA, Ashraf I (2012) Impact of foliar application of seaweed extract on growth, yield and quality of potato (*Solanum tuberosum* L.) Plant Soil Environ 31: 157–162, www.se.org.pk/File-Download.aspx?publishedid=154.

Hendrix JW (1993) Glomales fungi as plant pathogens. Mycorr News 5: 1–6.

Hepper CM, Azcon-Aguilar C, Rosendahl S, Sen R (1998) Competition between three species of glomus used as spatially separated introduced and indigenous mycorrhizal inocula for Leek (*Allium porrum* L.). New Phytol 110: 207–215.

Ignacimuthu S, Vendan SE (2007) Botanical pesticides in insect pest management. Uttar Pradesh J Zool 4: 141–154.

Jensen E (2004) Seaweed: Fact or Fancy? Organic Broadcaster 12: 164–170.

Karthikeyan K, Shanmugam M (2015) Yield and oil content of peanut (var. TMV-7) and sunflower (var. Co-2) applied with bio-stimulant AQUASAP manufactured from seaweed. Afr J Agr Res10: 2537–2543, http://dx.doi.org/10.5897/AJAR2015.9771.

Khang VT (2011) Fulvic foliar fertilizer impact on growth of rice and radish at first stage. Omonrice 18: 144–148, http://www.clrri.org/*ver2*/uploads/noidung/18–18.pdf.

Latique S, Chernane H, Mansori M, El Kaoua M (2013) Seaweed liquid fertilizer effect on physiological and biochemical parameters of bean plant (*Phaseolus vulgaris* variety paulista) under hydroponic system. Eur Sci J 9: 174–191, http://www.eujournal.org/index.php/esj/article/viewFile/1953/1895.

Latique S, Elouaer MA, Souguir M, Aloui H, Hannachi C, Chernane H, Mansori M, Elkaoua M (2014) Effect of seaweed extract of *Sargassum vulgare* on germination behavior of two bean cultivars (*Phaseolus vulgaris* L) under salt stress. IOSR J Agric Vet Sci 7: 116–120, http://www.iosrjournals.org/iosr-javs/papers/vol7-issue2/Version-1/T0721116120.pdf.

Lotfi R, Pessarakli M, Gharavi-Kouchebagh P, Khoshvaghti H (2015) Physiological responses of *Brassica napus* to fulvic acid under water stress: Chlorophyll a fluorescence and antioxidant enzyme activity. Crop J 3: 434–439, http://dx.doi.org/10.1016/j.cj.2015.05.006.

Lu LH, Wu OSh (2017) Mycorrhizas promote plant growth, root morphology and chlorophyll production in white clover. Biotechnology 16: 34–39, http://dx.doi.org/10.3923/biotech.2017.34.39.

Magarey RC, Bull JI, Reghenzani JR (2005) The influence of Vesicular Arbuscular Mycorrhizae (VAM) on sugarcane growth in the field. Proc Aust Soc Sug Cane Technol 27: 282–290, https://www.assct.com.au/media/pdfs/2005_Ag38_Magarey.pdf.

Malan C (2015) Review: humic and fulvic acids. A Practical Approach. In Sustainable soil management symposium. Stellenbosch, 5–6 November 2015, Agrilibrium Publisher.

Marhoon IA, Abbas MK (2015) Effect of foliar application of seaweed extract and amino acids on some vegetative and anatomical characters of two sweet pepper (*Capsicum Annuum* L.) cultivars. Int J Res Stud Agr Sci (IJRSAS) 1: 35–44, https://www.arcjournals.org/pdfs/ijrsas/v1-i1/5.pdf.

Matysiak K, Kaczmarek S, Krawczyk R (2011) Influence of seaweed extracts and mixture of humic and fulvic acids on germination and growth of *Zea mays* L. Acta Sci Pol Agricultura 10: 33–45.

Mohan JE, Cowden CC, Baas P, Dawadi A, Frankson PT, Helmick K (2014) Mycorrhizal fungi mediation of terrestrial ecosystem responses to global change: Mini-review. Fungal Ecol 10: 3–19, http://dx.doi.org/10.1016/j.funeco.2014.01.005.

Munda S, Shivakumar BG, Rana DS, Gangaiah B, Manjaiah KM, Dass A, Layek J, Lakshman K (2016) Inorganic phosphorus along with biofertilizers improves profitability and sustainability in soybean (*Glycine max*) – potato (*Solanum tuberosum*) cropping system. J Saudi Soc Agr Sci 17: 107–113, https://doi.org/10.1016/j.jssas.2016.01.008.

Pramanick B, Brahmachari K, Ghosh A (2013) Effect of seaweed saps on growth and yield improvement of greengram. Afr J Agr Res 8: 1180–1186, https://doi.org/10.5897/AJAR12.1894.

Prasad K, Das AK, Oza MD, Brahmbhatt H, Siddhanta AK, Meena R, Eswaran K (2010) Detection and quantification of some plant growth regulators in a seaweed-based foliar spray employing a mass spectrometric technique sans chromatographic separation. J Agric Food Chem 58: 4594–4601, https://doi.org/10.1021/jf904500e.

Roberts TL (2014) Cadmium and phosphorous fertilizers: the issues and the science. Procedia Engineer 83: 52–59, https://doi.org/10.1016/j.proeng.2014.09.012.

Sarhan TZ (2011) Effect of humic acid and seaweed extracts on growth and yield of potato plant (*Solanum tuberosum* l) desiree cv. Mesopotamia J Agric 39: 19–27, https://www.iasj.net/iasj?func=fulltext&aId=30377.

Schmidt W, Cesco S, Santi S, Pinton R, Varanini Z (2005) Water-extractable humic substances as nutrient acquisition signals for root hairs development in Arabidopsis. In: Hartmann A, Schmid M, Wenzel W, Hinnsinger P (eds) Rizosphere 2004 – Perspectives and Challenges. Neuherberg, Gsf-Berich.

Sutharsan S, Nishanthi S, Srikrishnah S (2014) Effects of foliar application of seaweed (*Sargassum crassifolium*) liquid extract on the performance of *Lycopersicon esculentum* Mill. In sandy regosol of batticaloa district Sri Lanka. Am Eurasian J Agric Environ Sci 14: 1386–1396, https://doi.org/10.5829/idosi.aejaes.2014.14.12.1828.

Wickham A, Davis JG (2015) Effect of liquid organic fertilizers and seaweed extract on *Daucus carota var. sativus* growth characteristics. Proceedings of the Western Nutrient Management Conference. Reno, Nevada, 5–6 March, 2015.

Williams A, Norton DA, Ridgway HJ (2012) Different Arbuscular mycorrhizal inoculants affect the growth and survival of *Podocarpus cunninghamii* restoration plantings in the Mackenzie Basin, New Zealand. New Zeal J Bot 50: 473–479, https://doi.org/10.1080/0028825X.2012.672429.

Wilson H, Johnson BR, Bohannan B, Pfeifer-Meister L, Mueller R, Bridgham SD (2016) Experimental warming decreases *arbuscular mycorrhizal* fungal colonization in prairie plants along a Mediterranean climate gradient. Peer J 4: e2083, https://doi.org/10.7717/peerj.2083.

Xu H, Lu Y, Tong S (2018) Effects of Arbuscular Mycorrhizal fungi on photosynthesis and chlorophyll fluorescence of maize seedlings under salt stress. Emir J Food Agric 30: 199–204, https://doi.org/10.9755/ejfa.2018.v30.i3.1642.

Xudan X (1987) The effect of foliar application of fulvic acid on water use, nutrient uptake and yield in wheat. Aust J Agric Res 37: 343–350, https://doi.org/10.1071/AR9860343.

Yamauchi M, Katayama S, Todoroki T, Watanable T (1984) Total Synthesis of Fulvic Acid. J Chem Soc, Chem Commun 23: 1565–1566, http://dx.doi.org/10.1039/C39840001565.

Yang S, Zhang Z, Cong L, Wang X, Shi S (2013) Effect of fulvic acid on the phosphorus availability in acid soil. J Soil Sci Plant Nut 13: 526–533, http://www.scielo.cl/pdf/jsspn/v13n3/aop4113.pdf.

Yigit F, Dikilitaş M (2008) Effect of humic acid applications on the root-rot diseases caused by *Fusarium spp*. on tomato plants. Plant Pathol 7: 179–182, https://doi.org/10.3923/ppj.2008.179.182.

Zare-Maivan H, Khanpour-Ardestani N, Ghanati F (2017). Influence of mycorrhizal fungi on growth, chlorophyll content and potassium and magnesium uptake in maize. J Plant Nutr 40: 2026–2032, https://doi.org/10.1080/01904167.2017.1346119.

Zhang X, Ervine EH (2004) Cytokinin-containing seaweed and humic acid extracts associated with Creeping bent grass leaf cytokinins and drought resistance. Crops 44: 1737–1745, https://doi.org/10.2135/cropsci2004.1737.

COMPARATIVE SUSCEPTIBILITY OF *CHIRONOMUS* AND *DROSOPHILA* TO EXPOSURE TO EACH AND COMBINATIONS OF THE FOLLOWING STRESSORS: DESICCATION, HEAT STRESS AND STARVATION

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ABSTRACT

In natural ecosystems, organisms are usually subject to environmental stress. In order to understand the response to a combination of three stressors (desiccation, heat stress and starvation), two dipteran insects, *Chironomus ramosus* (aquatic) and *Drosophila melanogaster* (terrestrial) were chosen, the former being more primitive than the latter. The mortality level as a function of the duration of the exposure to stress revealed that these two evolutionarily distinct and ecologically diverse insects differ in their response. Interestingly, when the tolerance thresholds of *C. ramosus* and *D. melanogaster* to single and multiple stressors was compared, a synergistic effect was recorded with much higher levels mortality occurring when subjected simultaneously to several stressors. *Chironomus* larvae were more vulnerable than *Drosophila* larvae when subjected to all three stressors simultaneously. The findings of this pilot study indicate the ecological risk for macro-invertebrate biota posed by adverse environmental conditions.

Keywords: *Chironomus*; desiccation stress; *Drosophila*; heat stress; multiple stress; starvation stress

Introduction

In nature, organisms experience changes in environmental conditions, such as heat stress, starvation, desiccation, osmotic imbalance, hypoxia, radiation, etc. Often, organisms exhibit specific patterns of adaptations in response to these environmental stresses (Bijlsma and Loeschcke 2005; Davies et al. 2014). In this context, insects are suitable model systems for determining the responses to environmental stress (Datkhile et al. 2015; Thorat et al. 2016, 2017). This is because in spite of being small and having a high metabolic rate, insects are among the most widely distributed animals and occur in almost all of the habitats in the biosphere and as a consequence are adapted to cope with a wide range of environmental stressors.

Effect of a combination of different stressors on organisms inhabiting diverse environmental conditions is of prime concern in the context of climate change. There is currently no in-depth study that address the adaptive response of insects to stressors. According to the 'Stress-Exposure-Response' (SER) model, abiotic stressors affect different species in variety of ways (Freedman 2015). Organisms have adapted to thrive and cope with multiple stressors acting simultaneously. The strategies used by insects to survive multiple stressors warrant more fundamental level studies, which is the rationale behind the present study. For this work, two phylogenetically distant and ecologically diverse insects, namely *Chironomus ramosus* and *Drosophila melanogaster,* were chosen. The former is an aquatic and the latter a terrestrial insect. We investigated the responses of these two insects to single and multiple stressors. Since survival depends on the length of exposure to stressors (Schulte 2014), we also looked into the temporal aspect of the response to stress. Our study revealed a striking difference in the responses to the exposure to specific stressors and several stressors simultaneously.

Methods

Rearing and maintenance of cultures

C. ramosus was mass reared in specially designed netted cages at a temperature of 24 ± 2 °C as described earlier (Nath and Godbole 1998). Early fourth instar larvae were used in all the experiments. An inbreed population of *D. melanogaster* (ORK strain) was maintained as described earlier by Thorat et al. (2016) in a BOD incubator set at 24 ± 2 °C.

Experimental Design

Ten larvae of either *D. melanogaster* or *C. ramosus* were used in each experiment, in which the larvae were exposed to either desiccation, heat stress or starvation, or combinations of these stressors for different durations or until 100% mortality was recorded. Each experiment was replicated ten times.

Desiccation

Five hundred grams of silica gel was added to the desiccating chamber 12 hrs prior to its use in order to obtain a value of <5% relative humidity (RH), which was monitored using a hygrometer. Larvae of *C. ramosus* and *D. melanogaster* were desiccated in this chamber on dry tissue paper placed in a glass Petri dish. The time to when

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100% had died was recorded. Larval survival was judged on the basis of either abdominal contractions (*Drosophila*) or undulatory movements (*Chironomus*) when gently poked with a blunt needle. Untreated larvae were used as a control. Each experiment was replicated ten times.

Heat stress

Heat stress was administered by transferring larvae of both *D. melanogaster* and *C. ramosus* to incubators at 37 °C and 40 °C, respectively, in order to subject them to heat stress. Induction of HSP70 as well as responses of *Chironomus* (Nath and Lakhotia 1989; Nath and Gharpure 2015) and *Drosophila* (Lindquist 1980) were the criteria used in the present study for indicating heat stress, which indicates it occurs at 40 °C in *C. ramosus* and 37 °C in *D. melanogaster*.

Starvation

Larvae of both *D. melanogaster* and *C. ramosus* were removed from the rearing media, carefully cleaned and then starved to death. Since Chironomid larvae need to be able to burrow into a substrate they were provided with a substrate consisting of inert sand particles as described by Naik et al. (2006).

Multiple stressors

Both larvae of *D. melanogaster* and *C. ramosus* were exposed to either desiccation (D), heat stress (H) or starvation (S) either in a combination of two (D + S; D + H; H + S) or three stressors (D + H + S) simultaneously. The time to when all the larvae had died was monitored for each combination.

Data analysis

Probit analysis of the percentage mortality data was carried out. The mean mortality ± SE values were analysed statistically followed by ANOVA. Percentage mortality of treated samples were normalized relative to that of the control (without stress) using Abbott's formula (Abbott 1925). The slope of the regression line resulting from the probit analysis was determined for each experiment and goodness of fit was assessed using Chi-square tests.

Results and Discussion

We determined the differences in the responses of larvae of both *D. melanogaster* and *C. ramosus* in terms of

Table 1 The LT_{20}, LT_{50} and LT_{90} values predicted by the Probit analysis of the results of different stress treatments: A) *Drosophila melanogaster*, B) *Chironomus ramosus*.

Stress	LT_{20} (Hrs)	Fiducial limits of LT_{20} value	LT_{50} (Hrs)	Fiducial limits of LT_{50} value	LT_{90} (Hrs)	Fiducial limits of LT_{90} value	Slope ± SE[b]	χ^2	d.f.[a]
A. *Drosophila melanogaster*									
Desiccation	8.092	7.520–8.707	9.494	8.823–10.016	11.391	10.889–11.767	12.131 ± 0.016	0.904	2
Heat stress	10.483	9.819–11.193	12.927	12.107–13.801	17.784	16.657–18.987	9.334 ± 0.015	0.994	9
Starvation	25.520	24.561–26.517	30.263	29.098–31.416	39.139	37.668–40.668	11.505 ± 0.008	1.000	20
Desiccation + Starvation	8.092	7.520–8.707	9.494	8.823–10.016	11.391	10.889–11.767	12.131 ± 0.016	0.904	2
Desiccation + Heat stress	3.428	3.045–3.860	4.387	3.897–4.940	6.388	5.673–7.193	7.931 ± 0.026	0.775	3
Heat stress + Starvation	8.502	8.064–9.385	11.004	10.198–11.873	15.733	14.580–16.977	8.304 ± 0.017	1.000	8
Multiple Stressors (D + H + S)	3.428	3.045–3.860	4.387	3.897–4.940	6.388	5.673–7.193	7.931 ± 0.026	0.775	3
B. *Chironomus ramosus*									
Desiccation	0.837	0.794–8.707	0.937	0.888–10.016	1.049	1.008–1.767	17.534 ± 0.016	0.884	3
Heat stress	14.691	13.735–15.712	22.319	20.868–23.871	42.193	39.450–45.127	4.679 ± 0.015	1.000	39
Starvation	49.778	48.229–51.378	62.899	60.940–64.922	89.820	87.022–92.708	8.323 ± 0.007	1.000	56
Desiccation + Starvation	0.837	0.794–8.707	0.937	0.888–10.016	1.049	1.008–1.767	17.534 ± 0.016	0.884	3
Desiccation + Heat stress	0.601	0.592–0.669	0.705	0.663–0.749	0.820	0.788–0.890	17.099 ± 0.014	0.804	2
Heat stress + Starvation	8.332	7.614–9.117	12.349	11.286–13.513	22.484	20.577–24.602	4.966 ± 0.020	1.000	19
Multiple Stressors (D + H + S)	0.601	0.592–0.669	0.705	0.663–0.749	0.820	0.788–0.890	17.099 ± 0.014	0.804	2

[a] Degrees of freedom; [b] Standard error; χ^2 = Chi-square value; LT_{20} = Length time required to kill 20% of the larvae; LT_{50} = Length of time required to kill 50% of the larvae; LT_{90} = Length of time required to kill 90% of the larvae.

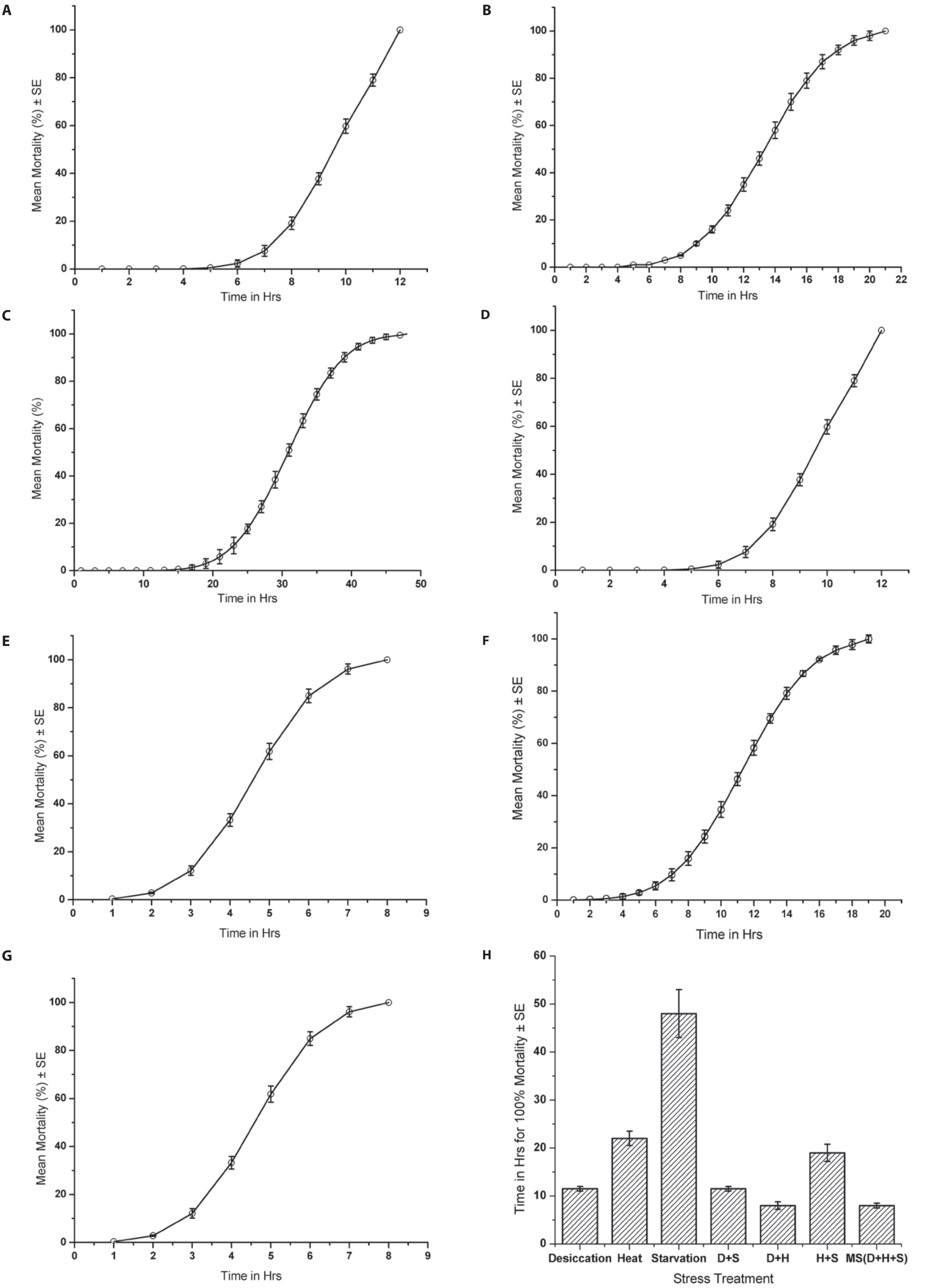

Fig. 1 Curve of the mean mortality recorded over time (hours) of *D. melanogaster* larvae exposed to a) desiccation, b) heat stress, c) starvation, d) desiccation + starvation, e) desiccation + heat stress, f) heat stress + starvation, g) multiple stressors: desiccation + heat stress + starvation, h) single and multiple exposure to stressors. F (6, 28) = 355.87, $P < 0.001$; ANOVA was used to compare the results for the different stress treatments.

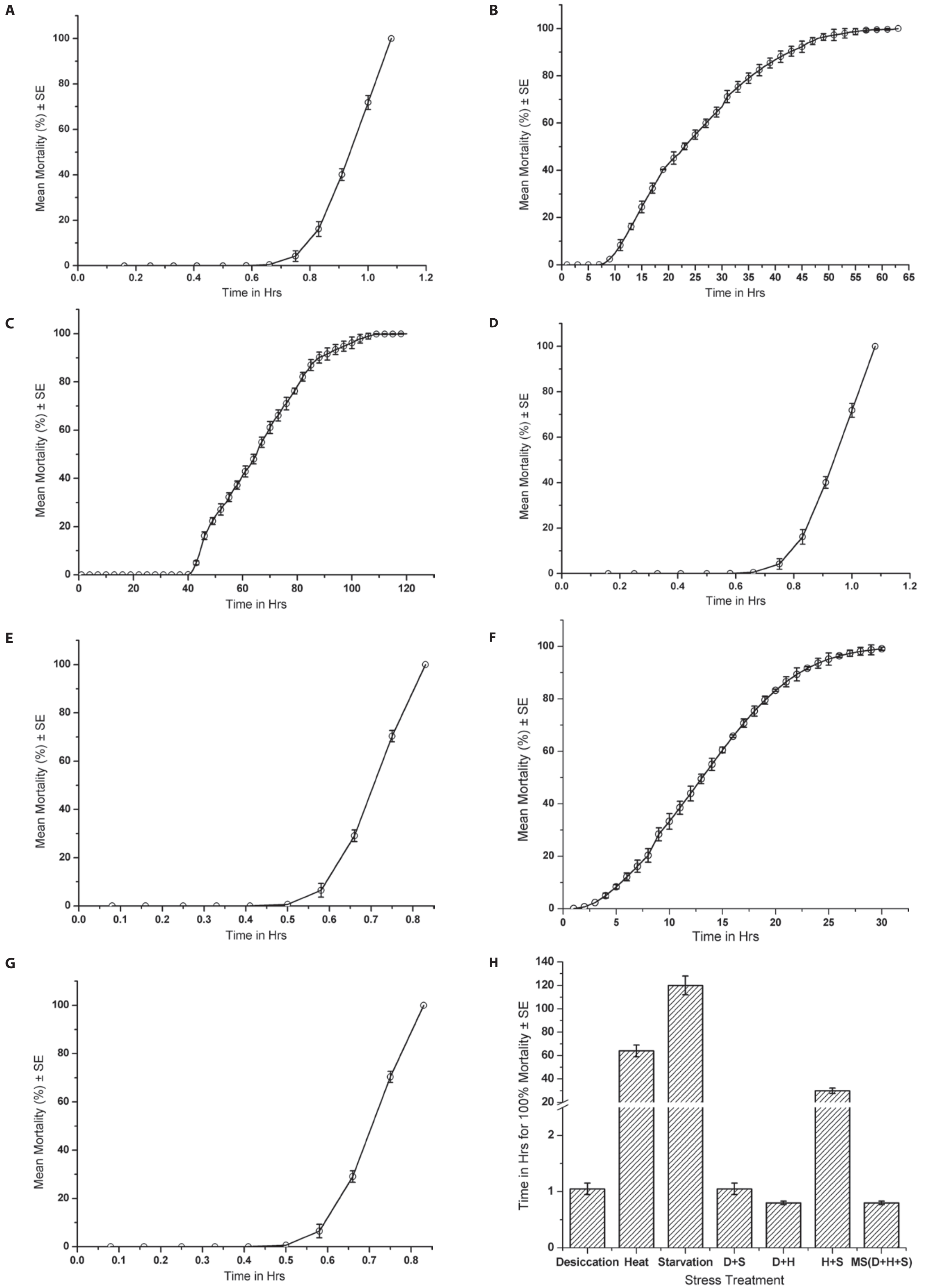

Fig. 2 Curves of the mean mortality recorded over time (hours) of *C. ramosus* larvae exposed to a) desiccation, b) heat stress, c) starvation, d) desiccation + starvation, e) desiccation + heat stress, f) heat stress + starvation g) multiple stressors: desiccation + heat stress + starvation, h) single and multiple exposure to stressors. $F (6, 28) = 2388.7$, $P < 0.001$; ANOVA was used to compare the results of the different stress treatments.

mortality due to desiccation, heat stress and starvation when subjected to these stressors separately or in combination. *D. melanogaster* showed a steady increase in morality with increase in the time the larvae were exposed to stress, irrespective of the type of stress. Its larvae survived an exposure to desiccation of 11.5 ± 0.5 hrs and 22 ± 1.5 hrs to heat stress and were even more tolerant of starvation as they survived for 48 ± 5 hrs. In the multiple stress treatments, they survived for11.5 ± 0.5 hrs in the D + S treatment, 8 ± 0.8 hrs in the D + H and 19 ± 1.8 hrs in the H + S treatment. The larvae of *D. melanogaster* were significantly less tolerant (8 ± 0.5hrs) of simultaneous exposure to several stressors, i.e. D + H + S, than a single stressor (Fig. 1). The time required to kill 20% (LT_{20}), 50% (LT_{50}) and 90% (LT_{90}) of the larvae was determined using Probit analysis (Table 1A).

The mortality of the larvae of *C. ramosus* also increased with the length of time they were exposed to the stressors. The larvae survived 1.05 ± 0.1 hrs of desiccation, 64 ± 5 hrs heat stress and 120 ± 8 hrs of starvation. In the multiple stress treatments, larvae survived 1.05 ± 0.1 hrs exposure to D + S, 0.8 ± 0.03 hrs to D + H and 30 ± 2.3 hrs to H + S. The larvae were significantly less tolerant of exposure to D + H + S, as they only survived for 0.8 ± 0.03 hrs (Fig. 2). Comparison of the results of single and multiple stress treatments revealed that the larvae of *C. ramosus* were significantly less tolerant of simultaneous exposure to several stressors than a single stressor. The time required to kill 20% (LT_{20}), 50% (LT_{50}) and 90% (LT_{90}) of the larvae was determined using Probit analysis (Table 1B).

Of the three stressors used, both *C. ramosus* and *D. melanogaster* were most vulnerable to desiccation followed by heat stress. This corroborates an earlier hypothesis that tolerance of desiccation determines the distribution of insects (Kellermann et al. 2009) along with the way insects cope with heat stress combined with a shortage of water (Chown et al. 2011). Nevertheless, starvation affected both the insects tested the least. Therefore, despite the differences in their ecology and evolution these two insects responded similarly to the stressors.

Interestingly, *D. melanogaster* was relatively more tolerant than *C. ramosus* when subjected to all three stressors simultaneously. This difference in the tolerance of terrestrial and aquatic insect of abiotic factors was previously reported by Chown et al. (2015). Nevertheless, aquatic insects experience less variation in their thermal environment than terrestrial insects, therefore, it is not surprising that aquatic insects are more sensitive to heat stress. Furthermore, desiccation affects aquatic Chironomid midge larvae more than the terrestrial Drosophilid larvae as is reported earlier by Thorat et al. (2017).

In the past, although concerns were raised about two-species comparisons (Garland and Adolph 1994), they were addressed by choosing model organisms with markedly different ecologies and using a more rigorous experimental protocol. Interestingly, although the organisms studied belong to same order they differ markedly in their ecology and evolutionary history.

According to Jorgensen (2010), organisms exposed simultaneously to multiple stressors may respond synergistically, additively or antagonistically. The findings of present study clearly indicate that the larvae of both *C. ramosus* and *D. melanogaster* show a synergistic response to a simultaneous exposure to several stressors, as such an exposure has a much greater effect than exposure to a single stressor.

A recently published meta-analysis of the status of multi-stress research on aquatic organisms indicates a lack of general consensus on their value in risk assessment and an appropriate scientific framework (Noges et al. 2016). In this context, our study is the first attempt, although on a small scale, to determine the effect on two species of insects of exposure to either a single stressor or a simultaneous exposure to several stressors.

Conclusions

The present study clearly indicate that the thresholds of tolerance of *C. ramosus* and *D. melanogaster* of desiccation, heat stress or starvation either on their own or combined differed. The effects of starvation, desiccation and heat stress are similar and future studies may throw light on the cellular and molecular basis of this commonality as well as the uniqueness of stress-signalling pathways when organisms are exposed simultaneously to many stressors. The results of this study provide a valuable insight into how to carry out ecological risk assessment programmes.

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REFERENCES

Abbott WS (1925) A method of computing the effectiveness of an insecticide. J Econ Entomol 18: 265–267.

Bijlsma R, Loeschcke V (2005) Environmental stress, adaptation and evolution: an overview. J Evol Biol 18: 744–749.

Chown SL, Sorensen JG, Terblanche JS (2011) Water loss in insects: an environmental changes perspective. J Insect Physiol 57: 1070–1084.

Chown SL, Duffy GA, Sorensen JG (2015) Upper thermal tolerance in aquatic insects. Curr Opin Insect Sci 11: 78–83.

Datkhile KD, Gaikwad PS, Ghaskadbi SS, Mukhopadhyaya R, Nath BB (2015) *Chironomus ramosus* larvae exhibit DNA damage control in response to gamma radiation. Int J Radiat Biol 91: 742–748.

Davies SA, Cabrero P, Overend G, Aitchison L, Sebastian S, Terhzaz S, Dow JA (2014) Cell signalling mechanisms for insect stress tolerance. J Exp Biol 217: 119–128.

Freedman B (2015) Ecological effects of environmental stressors. In: Oxford Research encyclopaedia of environmental science. doi: 10.1093/acrefore/978019.

Garland T Jr, Adolph SC (1994) Why not to do two-species comparative studies: limitations on inferring adaptation. Physiol Zool 67: 797–827.

Jorgensen SE (2010) Ecotoxicology. Academic Press, Oxford UK.

Kellermann V, Van-Heerwaarden B, Sgro CM, Hoffmann AA (2009) Fundamental evolutionary limit in ecological traits drive *Drosophila* species distribution. Science 325: 1244.

Lindquist S (1980) Varying pattern of protein synthesis in *Drosophila*: Implication for regulation. Dev Biol 77: 463–469.

Naik DG, Babrekar AA, Nath BB (2006) Pheromone-like compounds in the cuticle of aquatic *Chironomus* larva. Chem Ecol 22: 501–508.

Nath BB, Lakhotia SC (1989) Heat shock response in a tropical *Chironomus*: seasonal variation in response and the effect of developmental stage and tissue type on heat shock protein synthesis. Genome 32: 676–686.

Nath BB, Gharpure DG (2015) A novel computational approach of image analysis to heat shock in *Chironomus ramosus* larvae (Diptera: Chironomidae). Eur J Environ Sci 5: 57–61.

Nath BB, Godbole NN (1998) Technique for mass rearing of Indian Chironomus species (Diptera, Nematocera, Chironomidae). Stud Dipterol 5: 187–193.

Noges P, Argillier C, Borja A, Garmendia JM, Hanganu J, Kodes V, Pletterbauer F, Sagouis A, Birk S (2016) Quantified biotic and abiotic responses to multiple stress in freshwater, marine and ground water. Sci Total Environ 540: 43–52.

Schulte PM (2014) What is environmental stress? Insights from fish living in a variable environment. J Exp Biol 217: 23–34.

Thorat L, Oulkar D, Banerjee K, Gaikwad S, Nath BB (2017) High throughput mass spectrometry analysis revealed a role for glucosamine in potentiating recovery following desiccation stress in *Chironomus*. Sci Rep 7: 3659.

Thorat L, Oulkar DP, Banerjee K, Nath BB (2016) Desiccation stress induces developmental heterochrony in *Drosophila melanogaster*. J Biosci 41: 331–339.

OPTIMAL SCARIFICATION TIMES FOR SEEDS OF TWO MEDITERRANEAN ORCHIDS

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ABSTRACT

A critical step during the *in vitro* sexual propagation of Mediterranean orchids is the treatment of seeds with a disinfecting solution that also serves to scarify the seeds. If the seeds are not properly disinfected, microorganisms grow within the culture vessel, thus reducing the efficacy of the process in terms of the extra time and materials required. On the other hand, a long period of disinfection may damage the embryo. The literature is inconclusive with respect to the proper combination of disinfectant strength and duration of the treatment. The objective of this research is to determine the optimal scarification times for two orchid species with thin and thick seed coats, respectively. Seeds of *Anacamptis laxiflora* (Lam.) and *Himantoglossum robertianum* (Loisel.) were treated with 0.5% NaClO solution for 5, 15, 25, 35, 45, 55, 65, 75 and 85 minutes and sown in modified organic Malmgren medium. Logistic regression models were fitted to the results. We found that the longer the chemical treatment, the lower the percentage infection and higher the percentage germination, within the range of times tested. A chemical treatment of 85 minutes in 0.5% NaClO results in satisfactory percentage germination for both seeds with relatively permeable seed coats such as *A. laxiflora (*Lam.) and those with relatively impermeable seed coats such as *H. robertianum (*Loisel.).

Keywords: *Anacamptis laxiflora* (Lam.); Cephalonia; disinfection; *Himantoglossum robertianum* (Loisel.); *in vitro* propagation; Orchidaceae

Introduction

There are more than 200 species and subspecies of terrestrial orchids recorded in Greece (for example, Petrou et al. 2011; Dimopoulos et al. 2013; Antonopoulos and Tsiftsis 2017; Tsiftsis and Antonopoulos 2017). This remarkable biodiversity is threatened by diverse environmental pressures on their habitats such as cultivation and grazing (Follner et al. 1999; Gerakis et al. 2016; Hirth 2016), urbanisation and tourism (Kretzschmar et al. 2002), illicit collection (Kreziou et al. 2016) and climate change (Hutchings et al. 2018). Meanwhile, the horticultural, nutritional and medicinal value of native orchids remains relatively unexploited. *In vitro* propagation of native orchids would yield multiple benefits: protection and conservation of valuable genetic resources, the development of an innovative floricultural product for export, manufacture of salep and the possibility of restoring disturbed ecosystems. For this, it is desirable to develop a protocol for *in vitro* propagation suitable for Mediterranean native orchids that can be used by plant propagation laboratories and professionals for the mass production of seedlings for use in restoration programmes.

The seeds of many wild plants must undergo a process called scarification in order to break embryo dormancy and initiate germination. Scarification literally means "scratching". The first known use of the term is by the comedic playwright Aristophanes (*The Frogs*, 405 BCE), in reference to the writings of his contemporary philosophers who were ostensibly scribbling nonsense. Large seeds with hard coats often are physically scratched to facilitate imbibition of water. Orchid seeds are too small and fragile, so scarification involves a chemical treatment with a solution that serves two purposes: First, to disinfect the seed in preparation for sowing; second, to break embryo dormancy and initiate germination (Rasmussen 1995). If seeds are not properly disinfected, within a few days fungi, yeasts and occasionally bacteria grow within the culture vessels, reducing the efficacy of the process in terms of the extra time and materials needed. Too short a treatment may result in unduly high infection rates; too long a treatment may damage the embryo. The decision of how long to scarify seeds is further complicated by the fact that different species have seed coats (testas) of different thickness, necessitating different treatment times (Malmgren and Nyström 2018).

Scarification of terrestrial orchid seeds is poorly documented and the sparse evidence is contradictory. For instance, Malmgren and Nyström (2018) recommend soaking the seeds in 0.3–1% NaClO solution for 5–45 minutes. On the contrary, Rasmussen (1995) suggests a stronger NaClO solution (5%) and much longer treatment times, up to several hours. Further details of the scant literature are provided by Katsalirou et al. (2017). The corollary is that, if mass Mediterranean orchid propagation is ever attempted for commercial or conservation purposes, optimal scarification times must be empirically determined for each species.

Having acquired general experience of *in vitro* culture of Mediterranean orchids, we initiated a formal experiment to determine optimal seed disinfection times for two species, one with a relatively permeable seed coat, *Anacamptis laxiflora* (Lam.), and one with a relatively impermeable seed coat, *Himantoglossum robertianum* (Loisel.). The objective was to determine treatment times that minimize infection of culture vessels and maximize

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seed germination without unduly delaying the *in vitro* procedure. The first results were reported in Katsalirou et al. (2017). Due to the small number of replications per treatment (n = 8), the predictive ability of regression models of percentage infection on treatment duration was no better than the mean percentage infection. However, regression models of percentage germination on treatment duration proved statistically significant or nearly so. Scarification times of only a few minutes in 1% NaClO seemed optimal for germination of seeds with relatively permeable seed coats such as those of *Anacamptis laxiflora* (Lam.). In contrast, scarification times of as long as 45 minutes in 1% NaClO were not long enough for seeds with relatively impermeable seed coats such as those of *Himantoglossum robertianum* (Loisel.). The lessons learned from that study were:

a) Eight replications are too few to obtain statistically significant results.
b) For seeds with relatively permeable coats, a weaker NaClO solution is better for controlling the scarification process as the treatment is more prolonged.

In this study, we repeat the experiment with more replications per treatment, weaker NaClO solution and longer treatment times.

Materials and Methods

Preparation of disinfecting solution

As a stock solution, we used a commercial formulation of household bleach (Klinex®, Unilever) with a nominal NaClO concentration of 4.8% w/w. To verify the strength of the formulation, we titrated three different volumes (5, 10, and 20 mL) with $AgNO_3$ solution following Mohr's method (Harris 2010). The manufacturer's reported concentration was found to be accurate. The stock solution was diluted with deionised water to a final concentration of 0.5%. Four drops of dish detergent per litre were added to reduce surface tension.

Experimental treatments

Combinations of nine treatment durations (5, 15, 25, 35, 45, 55, 65, 75 and 85 minutes) and two orchid species were tested. The two species were selected based on the permeability of their seed coats; a species with a relatively permeable seed coat, *Anacamptis laxiflora* (Lam.), and a species with a relatively impermeable seed coat, *Himantoglossum robertianum* (Loisel.).

In a proper factorial experiment, combinations of treatments are assigned to culture vessels at random. However, this means we would have to keep track of nine different treatment durations per experimental run, unduly increasing the complexity of the experimental setup and the possibility of operator error. Instead, we tested one treatment duration per experimental run, in reverse order; the longest duration was tested first to minimize the effect of operator learning. Had we proceeded the opposite way, we would give undue credit to the longest duration as a result of the operator improving his or her technique. Although operator experience can be an important factor in specialized work, such as *in vitro* propagation, this factor should best be tested in a separate experiment.

Each experimental run consisted of 24 culture vessels per species that served as experimental replications plus eight "blanks", i.e., culture vessels without seed. The blanks were subject to the same treatment as the vessels with seed, including simulated sowing with imaginary seed. The purpose of the blanks was to reveal possible shortcomings of our technique that were not due to seed-borne contaminants.

Field method

The seeds were collected on the island of Cephalonia, Greece. At flowering, healthy and robust individuals of *Himantoglossum robertianum* (Loisel.) and *Anacamptis laxiflora* (Lam.) were identified in the field, photographed, labelled and recorded. At senescence, seed was collected from mature capsules. Both species are abundant enough so that seed collection did not jeopardize the natural populations. The seeds were sieved to remove foreign matter such as capsule fragments, dried in a desiccator with silica gel, sealed in glass vials and stored at −20 °C until sowing.

Laboratory method

In nature, germinating orchid seeds develop a symbiotic association with fungi that supply them with nutrients. The seeds have almost no nutrient reserves of their own, so that germination depends on a fungus-orchid association called mycorrhiza (Garbaye 2013). It is possible to reproduce the mycorrhizal association in the laboratory. Although this approach most closely mimics nature, it is complicated because the operator must manage two organisms instead of one. Instead, many practitioners opt for a sterile nutrient solution that supplies the seed with the nutrients necessary for germination and early development.

Preparation of nutrient medium

There exist numerous recipes for nutrient media for propagating terrestrial orchids (Rasmussen 1995). A few base ingredients are included in most recipes, which makes sense, as most terrestrial plants require the same 16 nutrients for growth and development (Marschner 1995). Some recipes add unusual elements such as Al and I, the benefit of which has not been unequivocally demonstrated; others add tropical fruit juices or tree sap, the role of which has yet to be explained. We opted for a modified version of "SM-organic" (Rasmussen 1995), a time-tested formula used by veteran practitioner Svante Malmgren. The medium is termed "organic" on account of its N source, an amino acid mixture sold under the trade name Vaminolac®. However, in Greece

Table 1 Formula for modified "SM-organic" nutrient medium.

Ingredient	Quantity
Mineral water to make	1 l
$CaHPO_4$	99 mg[a]
KH_2PO_4	75 mg
$MgSO_4 \cdot 7H_2O$	75 mg
Soluvit (water soluble vitamins)	10 ml
Amina-Fe	0.92 ml[b]
Kinetin	5 mg
Saccharose (sucrose)	10 g
Activated charcoal, powdered	1 g
Danish agar	12 g
Pineapple (*Ananas comosus*) juice	25 ml
NH_3 or HCl for pH adjustment	1–2 drops
Potato (*Solanum tuberosum*) tuber	1 cm^3 per culture vessel

[a] Equivalent to 75 mg $Ca_3(PO_4)_2$ in the original formula.
[b] Equivalent to 0.5 ml Vaminolac® plus 10 mg $FeSO_4$ in the original formula.

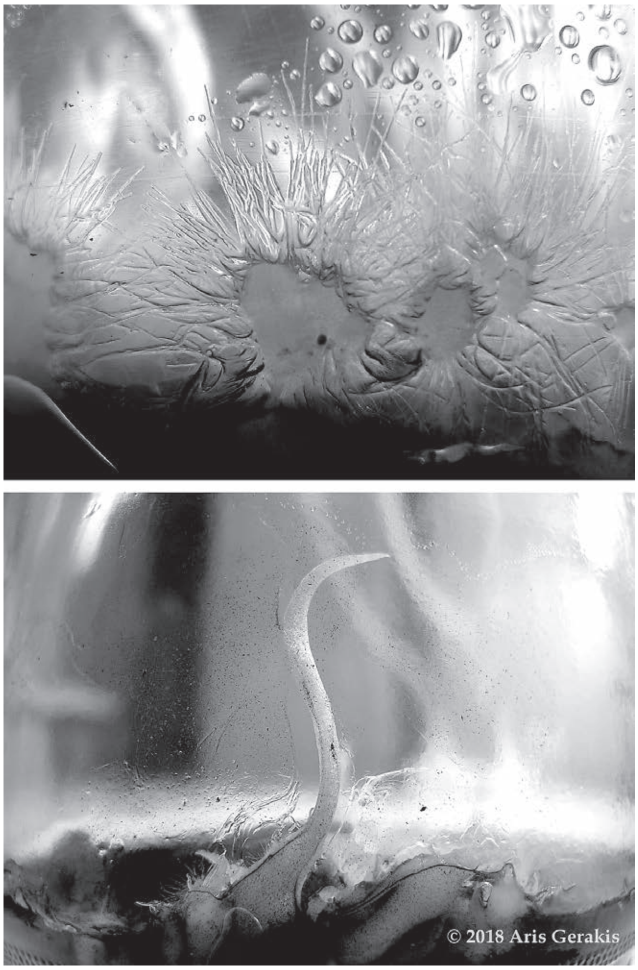

Fig. 1 Photographs of germinated seed: (a) *Anacamptis laxiflora* (Lam.), a few weeks after sowing and (b) *Himantoglossum robertianum* (Loisel.), a few months after sowing.

Vaminolac® is considered a medicine and as such cannot be sold without physician's prescription. Therefore, we substituted Amina-Fe (Humofert), a liquid fertiliser containing amino acids and chelate Fe. Because the addition of Amina-Fe more than covers the Fe requirement in Malmgren's recipe, we omitted the ferrous salt of the original formula. Further, we substituted $CaHPO_4$ for $Ca_3(PO_4)_2$. Mineral water was substituted for tap water, on account of the high salinity of the municipal water supply (electrical conductivity at 25 °C > 1 mS cm^{-1}). The formula is in Table 1.

The ingredients were mixed in a 1 l Erlenmeyer flask placed on a hot plate with a magnetic stirrer. After the solution cooled to ambient temperature, pH was adjusted to 5.5–6.0 with either NH_3 or HCl.

Preparation of materials

The nutrient medium was evenly distributed to 250 ml Erlenmeyer flasks for autoclaving. Glassware, steel tools, filter paper, potato cubes and deionized water were also sterilized in the autoclave, a Tuttnauer 2340 programmed on a 30 minute cycle (for cold departure) or a 25 minute cycle (for hot departure) at a temperature of 121 °C and pressure of 1.2 bar.

The sowings were carried out inside an Esco EQU/04-EBC-2A laminar flow cabinet. The interior surfaces were wiped with cotton wool soaked in 70% v/v ethanol. The culture vessels were 100 mL urine samplers made of polypropylene, individually wrapped in sterile packaging. All non-autoclavable materials were placed in the laminar flow cabinet and exposed to a germicidal UV lamp for 50 minutes. The workspace outside the laminar flow cabinet was also disinfected with a germicidal UV lamp for 50 minutes. The airflow in the cabinet was turned on for 15 minutes to purge airborne contaminants as per manufacturer's instructions.[1] The sterile seal of the culture vessels was broken. The Erlenmeyer flasks with the nutrient medium were transferred from the autoclave onto a hotplate inside the laminar flow cabinet with the thermostat set to 50 °C.

Seed Disinfection

The seeds were disinfected in glass test tubes sealed with a cap. Ninety mg of seed was scooped up with a spatula and placed in each tube, except those assigned as blanks. The test tubes were filled nearly to the top with disinfecting solution, capped and shaken vigorously to remove any air bubbles in contact with the seed. The shaking was repeated every 15 minutes thereafter till the end of the disinfection period.

At the end of the disinfection period, the suspensions were decanted into Erlenmeyer flasks fitted with a polypropylene funnel lined with filter paper to recover the

1 Esco®. Class II Type A/B3 Biohazard Safety Cabinets. User Operation Manual.

seed. The filter paper was soaked in the same disinfecting solution as the seed. After filtering the suspensions, each filter paper was rinsed five times with approximately 7 ml of sterile deionized water.

Sowing

Each culture vessel was filled with approximately 17 ml of sterile nutrient medium and a potato cube. Once the nutrient medium cooled down to about 35 °C, the seeds were scraped from the filter paper using a spatula and distributed in the culture vessels. The tips of the steel tools were heat sterilized between sowing.

The culture vessels were incubated in a dark cabinet at an ambient temperature of 22 °C. Forty one days after the final sowing, the vessels were visually examined for the development of fungus, yeast and bacterial colonies. The infected vessels were counted and the non-infected vessels were returned to the cabinet. After 189 days, the vessels with germinated seeds were counted. Fig. 1 shows the germinated seeds of the two species.

Statistical methods

Because the response variables are nominal (infected vs. non-infected, germinated vs. non-germinated), one way to analyse the responses is by logistic regression (SAS Institute 2003). The probability of a nominal response with two levels (e.g., germinated vs. non-germinated) is modelled as a function of a regressor (in this case, treatment duration):

$$\%P\ (Y = 1\text{st response}) = 1 / [1 + e^{-(\beta_0 + \beta_1 X)}] \times 100 \qquad (1)$$

$$\%P\ (Y = 2\text{nd response}) = 100 - \%\ P(Y = 1\text{st response}) \qquad (2)$$

where %P is percent probability, Y = nominal response, X = regressor and β_0, β_1 = fitting parameters. The level of significance for statistical tests was set *a priori* at $\alpha = 0.05$.

Results and Discussion

The infection rate for the 72 "blank" vessels was nil, which proves that our technique was valid and that all contamination came from the seed. The percentage germination for the 72 "blank" vessels also was nil, naturally. As far as the vessels with seed, we noticed that the seed in any vessel that was not infected, germinated. This greatly simplified subsequent calculations, because all we had to model was either the percentage infection or the percentage germination for any given treatment, as they summed up to 100%. Of course, at the end of the day what matters most is percentage germination, because that determines how many useable seedlings are produced. Percentages germination for the two species are compared in Fig. 2. Logistic regression models were significant for both species. The statistical output is in Table 2.

The models of percentage germination in Fig. 2 are derived from eq. (1) substituting the parameter estimates from Table 2. To model percentage infection, we reverse the sign of the parameters, i.e., $\beta_0 = 2.6647$, $\beta_1 = -0.0574$ for *A. laxiflora* and $\beta_0 = 1.2824$, $\beta_1 = -0.0344$ for *H. robertianum*.

A notable result is that a weaker NaClO solution (0.5 as opposed to 1%) affords better control of the scarification process of seeds with a relatively thin testa such as those of *A. laxiflora*. Seeds with thin testa imbibe solution fast. Katsalirou et al. (2017) demonstrate that seeds of *A. laxiflora* can only be kept for a few minutes in 1% NaClO solution before their viability is reduced. However, too short a treatment may cause the operator to rush, increasing the possibility of error. In addition, it raises the question whether the time it takes to fill up, agitate and empty the test tubes containing the disinfectant solution should count toward the time necessary for scarification. For the longer treatment times afforded by weaker solutions, the answer is not critical.

Table 2 Model test and parameter estimates for logistic regression of percentage germination on treatment duration: LL = log likelihood, DF = degrees of freedom, χ^2 = chi-square, p = probability, R^2 = ratio of "Difference" to "Reduced" LL, β_0 and β_1 = fitted parameters.

Whole Model Test								
	Anacamptis laxiflora (Lam.)				*Himantoglossum robertianum* (Loisel.)			
Model	–LL	DF	χ^2	$p > \chi^2$	–LL	DF	χ^2	$p > \chi^2$
Difference	40.04	1	80.08	<.0001	17.91	1	35.83	<.0001
Full	109.60				130.47			
Reduced	149.64				148.38			
R^2	0.27				0.12			
Observations	216				216			
Parameter Estimates for Log Odds of P(germinated) / P(non-germinated)								
Term	Estimate	SE	χ^2	$p > \chi^2$	Estimate	SE	χ^2	$p > \chi^2$
Intercept (β_0)	–2.6647	0.39	45.82	< .0001	–1.2824	0.30	17.75	< .0001
Duration (β_1)	0.0574	0.01	54.47	< .0001	0.0344	0.01	30.54	< .0001

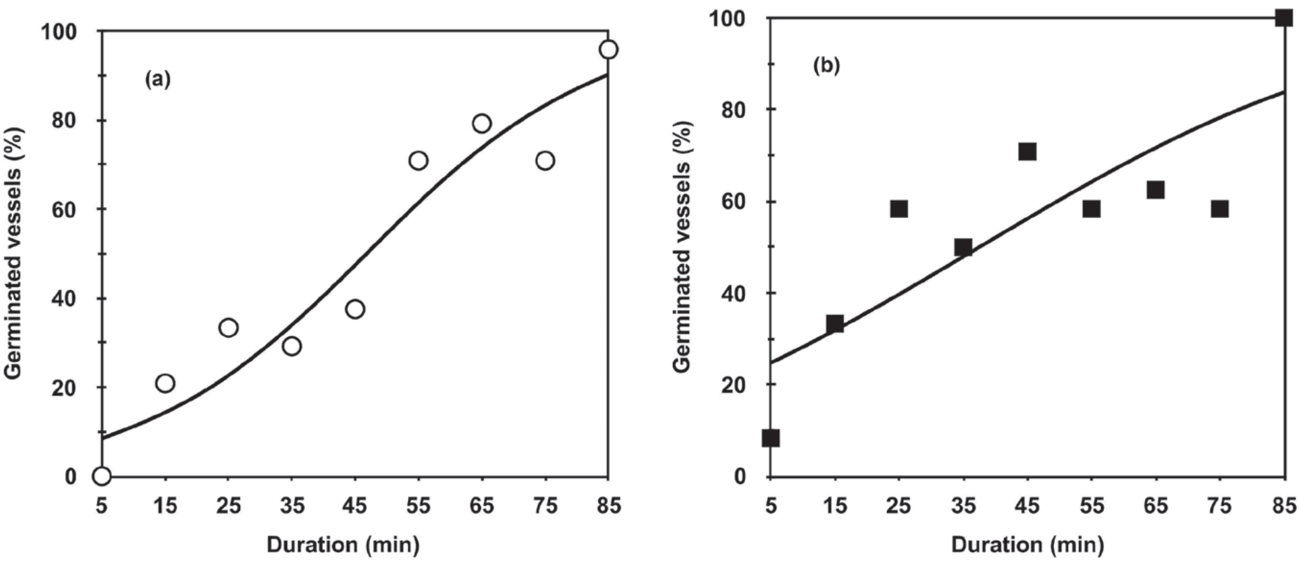

Fig. 2 Effect of treatment duration on percentage germination of (a) *Anacamptis laxiflora* (Lam.) and (b) *Himantoglossum robertianum* (Loisel.), 189 days after sowing. Markers are measured points (n = 24) and curves are fitted models. The models are derived from eq. (1) substituting parameter estimates from Table 2.

From Fig. 2 it follows that the longer the chemical treatment, the lower the predicted percentage infection and the higher the predicted percentage germination, within the range of times tested. A chemical treatment of 85 minutes in 0.5% NaClO results in satisfactory predicted percentage germination for both species, namely 90% for *A. laxiflora* and 84% for *H. robertianum*. Prolonging the treatment beyond 85 minutes will not necessarily improve percentage germination and in fact may reduce seed viability. In this sense, while regression models can be useful to illustrate the difference in scarification times among various species, they should not be extrapolated outside the range of tested times to avoid unwarranted conclusions.

In light of these results and those of our earlier study (Katsalirou et al. 2017), the advice of Malmgren and Nyström (2018) to soak terrestrial orchid seeds in 0.3–1% NaClO solution for 5–45 minutes is only a general recommendation. We found that a few minutes more or less can make the difference between a successful and an unsuccessful sowing. Rasmussen's (1995) recommendation of a stronger NaClO solution (5%) and treatment of up to several hours for *Dactylorhiza maculata* and *Epipactis helleborine* would probably harm the seeds of the two species that we tested. In conclusion, there is no easy way to predict optimal scarification times for seeds of terrestrial orchids, short of testing each species individually.

Artificial propagation of native orchids can be helpful to conserve populations threatened by extinction, especially stenoecious taxa, i.e., those confined to a rare habitat. Once the habitat is lost, so is the population. The first step in a conservation project would be to identify populations at risk and collect seed to establish a seed bank. The second step would be to cultivate plants from seed with a two-fold purpose: (a) to further multiply the seed and (b) to re-establish plants at suitable locations. Possible fields of application include: (a) restoration of disturbed habitats, such as quarries, mines and road banks, (b) mitigation of environmental effect of public works, (c) preservation of populations in coastal habitats (e.g., sand dunes) threatened by sea level rise, and (d) enrichment of orchid collections of botanical gardens.

Conclusions

Chemical scarification of seeds is a critical step in the *in vitro* propagation of Mediterranean orchids. On the one hand, too short a treatment may result in a high percentage of infected vessels, which translates into a waste of labour and material resources. On the other hand, unduly long treatments slow down the procedure and may reduce seed viability. Empirically derived regression models are useful for illustrating the difference in the scarification requirements of various species. In our case, a scarification time of 85 minutes in 0.5% NaClO resulted in a satisfactory predicted percentage germination for both seeds with relatively permeable seed coats (*Anacamptis laxiflora* Lam.) and those with relatively impermeable seed coats (*Himantoglossum robertianum* Loisel.). For statistically significant results, the appropriate number of replicates need to be determined; 24 replicates per treatment proved adequate for this study.

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REFERENCES

Antonopoulos Z, Tsiftsis S (2017) Atlas of the Greek Orchids. Vol. II. Mediterraneo Editions, Rethymno.

Dimopoulos P, Raus T, Bergmeier E, Constantinidis T, Iatrou G, Kokkini S, Strid A, Tzanoudakis D (2013) Vascular plants of Greece: an annotated checklist. Botanic Garden and Botanical Museum Berlin-Dahlem, Freie Universität Berlin and the Hellenic Botanical Society, Athens.

Follner K, Antzoulatos Y, Blank S, Klarenberg A, Panou A, Mommertz S (1999) Effects of grazing on the *Abies cephalonica* (Loudon) forest of Mount Ainos (island of Cephalonia, Ionian Sea, Greece). Contributions to the Zoogeography and Ecology of the Eastern Mediterranean Region 1: 83–88.

Garbaye J (2013) La symbiose mycorhizienne: une association entre les plantes et les champignons. Editions Quae, Versailles.

Gerakis A, Haldas X, Giannakoulias M (2016) *Anacamptis palustris* subsp. *robusta* (Orchidaceae): a new record to the flora of Cephalonia. Greece J Eur Orch 48: 11–18.

Harris DC (2010) Quantitative chemical analysis. 8th ed. W. H. Freeman, San Francisco, CA.

Hirth M (2016) A long-term survey of orchids on the small Greek Island Agathonisi from 1994 to 2013. J Eur Orch 48: 37–52.

Hutchings MJ, Robbirt KM, Roberts DL, Davy AJ (2018) Vulnerability of a specialized pollination mechanism to climate change revealed by a 356-year analysis. Bot J Lin Soc 186: 498–509, https://doi.org/10.1093/botlinnean/box086.

Katsalirou E, Gerakis A, Haldas X, Deconninck G (2017) Optimal disinfection times for seeds of Mediterranean orchids propagated on nutrient media. Eur J Environ Sci 7: 119–124, https://doi.org/10.14712/23361964.2017.10.

Kretzschmar H, Kretzschmar G, Eccarius W (2002) Orchideen auf Kreta, Kasos und Karpathos: Ein Feldführer durch die Orchideenflora der zentralen Inseln der Südägäis. Selbstverlag H. Kretzschmar. Bad Hersfeld.

Kreziou A, De Boer H, Gravendeel B (2016) Harvesting of salep orchids in north-western Greece continues to threaten natural populations. Oryx 50: 393–396, https://doi.org/10.1017/S0030605315000265.

Malmgren S, Nyström H (2018) Orchid propagation. http://www.lidaforsgarden.com/orchids/engelsk.htm. Accessed 4 Dec 2018.

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, San Diego, CA.

Petrou N, Petrou M, Giannakoulias M (2011) Orchids of Greece. Koan/Eight Clouds, Athens. Rasmussen HN (1995) Terrestrial orchids: From seed to mycotrophic plant. Cambridge U. Press, Cambridge.

SAS Institute (2003) JMP statistics and graphics guide. Version 5.1.2. SAS Institute, Cary, NC.

Tsiftsis S, Antonopoulos Z (2017) Atlas of the Greek Orchids. Vol. I. Mediterraneo Editions, Rethymno.

HOW MUCH IS APHID POPULATION DYNAMICS AFFECTED BY THEIR NATURAL ENEMIES? AN EMPIRICAL EXAMPLE FROM GREECE

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ABSTRACT

By monitoring of 50 aphid colonies in 2017 and another 50 colonies in 2018 twice a week, we looked at how the aphid dynamics was affected by their natural enemies. This will help us to see, how much the presence of natural enemies shortens the duration of an aphid colony, which may be one of the causes, why *Harmonia axyridis* is not very successful in the Mediterranean: the aphid colony may exist for a period of time too short in the Mediterranean conditions to enable successful development of *H. axyridis*.

Keywords: Aphids; population dynamics; *Harmonia axyridis*; colony

Introduction

Invasive alien species belong to the major drivers of biodiversity loss (Hilton-Taylor 2000). The harlequin ladybird (*Harmonia axyridis*) is an invasive alien species, which has probably escaped from the glasshouses in the Netherlands, country of its initial wild occurrence in Europe, and is now quickly spreading all across Europe (Brown et al. 2011; Roy et al. 2012). During the last decades, its distribution was reported from some countries very far from the place of its initial spread: from Ukraine, Turkey or Georgia (Roy et al. 2016). It is therefore interesting to see, what is the distribution and dynamics of aphidophagous guilds and aphids in these "distant" countries.

From the spreading of *H. axyridis* some practical and interesting question have arisen: 1) Will *Harmonia axyridis* suppress the abundance of native species of ladybirds? 2) What will be the results of *H. axyridis* arrival for aphid biocontrol?

Here we present results of a pilot study performed in one site in Greece as an example of a destination at the margin of *H. axyridis* distribution, where this species is still absent or is present only in small numbers. We looked at the structure and dynamics of aphidophagous guilds and aphids here, with a special focus on the dynamics and distribution of *H. axyridis*. We were not able, however, to measure any functional responses *sensu* Pervez et al. (2018) or Pervez and Yadav (2018), as the data were too scarce for this.

Methods

The data was collected in north-eastern Greece around Néa Péramos village, which is situated on the coast (Fig. 1).

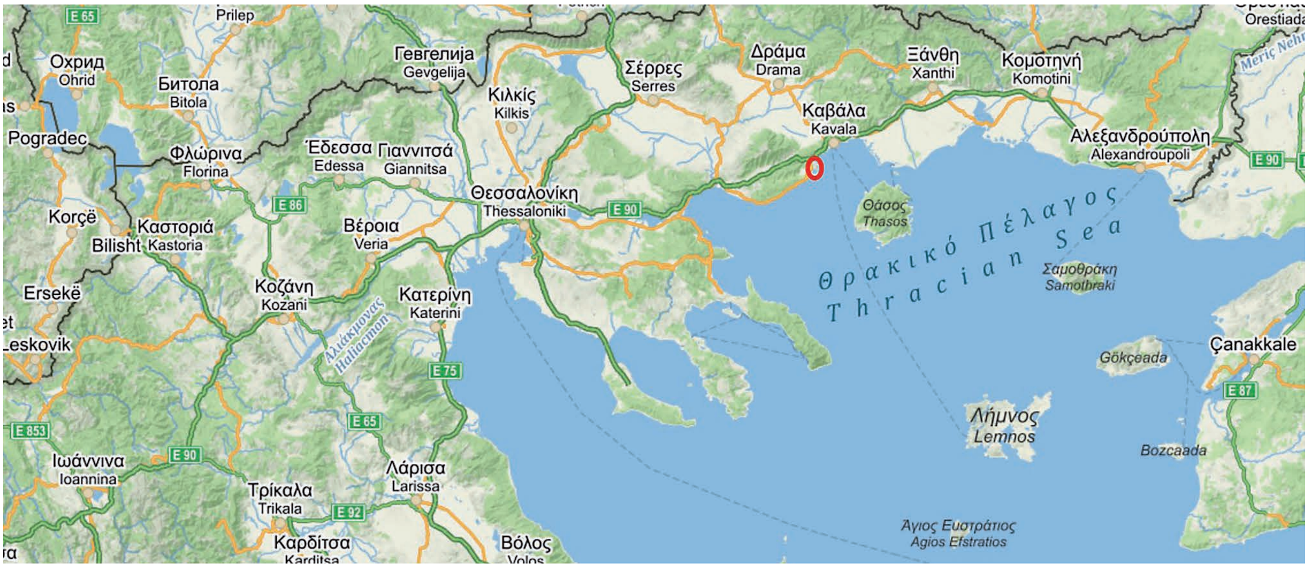

Fig. 1 Location of the study sites. The red ring shows where Néa Péramos is situated.

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We spent there a total of five months during 2017 and 2018, each year from April to mid of June, when aphid colonies occurred there.

In the field, we looked for aphid colonies on different plants around the village and when we found a suitable colony, we marked it by GPS and attached a colour label to the plant. In every aphid colony, we monitored the numbers of aphids and ladybirds (larvae, pupae and adults), other aphid enemies and also any kinds of changes within the colony. We excluded trees from our research in 2018, because of possible sampling error and because in 2017 we observed that aphids stayed on trees during the whole season, which made considerations of the colony duration irrelevant.

We visited marked colonies every 3 days and counted the aphids and their enemies on the whole plant. For making it a bit easier, especially in the case of trees, we tried to choose only smaller individuals of plants – smaller trees, single standing raspberry sprouts and so on. In total, we monitored 50 aphid colonies in 2017 and another 50 colonies in 2018. The monitoring was performed twice a week during the whole season.

To describe aphid population dynamics, we used the population dynamics models described by Kindlmann et al. (2004) and Kindlmann and Dixon (1999). The rate of change of aphid abundance is described as exponential growth of the aphid population and density dependent factor, where, instead of carrying capacity used in logistic equation, we used aphid cumulative density – the cumulative number of aphids from the beginning of colony existence:

$$x'(t) = r\,x(t)\left[1 - \alpha \int_0^t x(\tau)d\tau\right] \qquad (1)$$

Here $x(t)$ means number of aphids at time t, $x'(t)$ means change of the number of aphids per unit time at time t, r means instantaneous growth rate of the aphid population (in exponential growth), α means intensity of regulation (scaling constant, inversely proportional to carrying capacity of the plant) and $\int_0^t x(\tau)d\tau$ means cumulative number of aphids from the beginning of the colony existence.

Sometimes we had to estimate the exact arrival date of aphids in case that there were already more aphids when we first observed the colony. Then the dynamics was simulated backwards by an exponential. For the estimation of parameters, we used Euler method (minimum residual sum of squares) and module Solver in Excel to get parameters a and r.

Fig. 2 Ladybird species observed during 2017 and 2018. (a) *Adalia* sp., (b*) Coccinella septempunctata*, (c) *Hippodamia variegata*, (d) *Thea 22-punctata*. For *Harmonia axyridis* see Fig. 3.

Results and Discussion

We observed frequent diversions in aphid population dynamics from the predictions of model (1): both natural and human-caused ones. Natural diversions were mainly caused by aphids being fed by their enemies (ladybirds in most cases). Diversions due to human influence (often causing destruction of the whole colony) were a consequence of mowing or animal (mainly goat) grazing. We were not able to explain the reasons for diversions in some colonies. Sometimes the reason for diversion might have been due to sampling error, small numbers of aphid individuals or even unknown events.

Vast majority of aphid natural enemies consisted of ladybird larvae or adults, followed by hoverfly larvae and parasitoid wasps from the subfamily Aphidiinae. During our observations, we determined the following ladybird species visiting aphid colonies: *Adalia* sp., *Coccinella septempunctata*, *Harmonia axyridis* (*conspicua, spectabilis* and *succinea* forms), *Hippodamia variegata* and *Thea 22-punctata* (Figs. 2 and 3).

When trees were sampled in 2017 (Fig. 4), ladybirds (both larvae and adults) were dominated by *H. axyridis* (larvae 86.4%; adults 70.6%), followed by some individuals of *C. septempunctata* (larvae 13.6%; adults 27.6%). In 2018 (Fig. 5), however, when we excluded trees from the sampling, the aphidophagous guilds were dominated by *C. septempunctata* (larvae 93.8%; adults 64.7%) followed by some individuals of *Hippodamia variegata* (23.5%). We did not observe any adults of *Harmonia axyridis* in 2018.

In 2017, *H. axyridis* larvae were mainly present on tree species (like apple, peach and *Prunus* trees) while *C. septempunctata* larvae were present also on herbaceous plants. Regarding adults, the pattern was almost the same in the case of *H. axyridis* but we found *C. septempunctata* adults on trees, as well as on herbaceous plants at a higher rate (Fig. 6).

In 2018, the highest abundance of ladybird larvae and adults was observed on *Rumex* sp. and partly also on *Sonchus* sp. (mainly *C. septempunctata*); we also found some adults of *Hippodamia variegata*, *Thea 22-punctata* and *Adalia* sp. When only herbaceous plants in both years are considered, ladybirds were present mainly on *Rumex* and partly also on *Sonchus* (Fig. 7).

We also looked at how much the presence of natural enemies shortens the duration of an aphid colony, using eq. (1). According to the theory we tested, this may be

Fig. 3 Forms of *Harmonia axyridis* observed: (a) f. *conspicua*, (b) f. *spectabilis*, (c) f. *succinea*, (d) f. *axyridis* (not observed during this study).

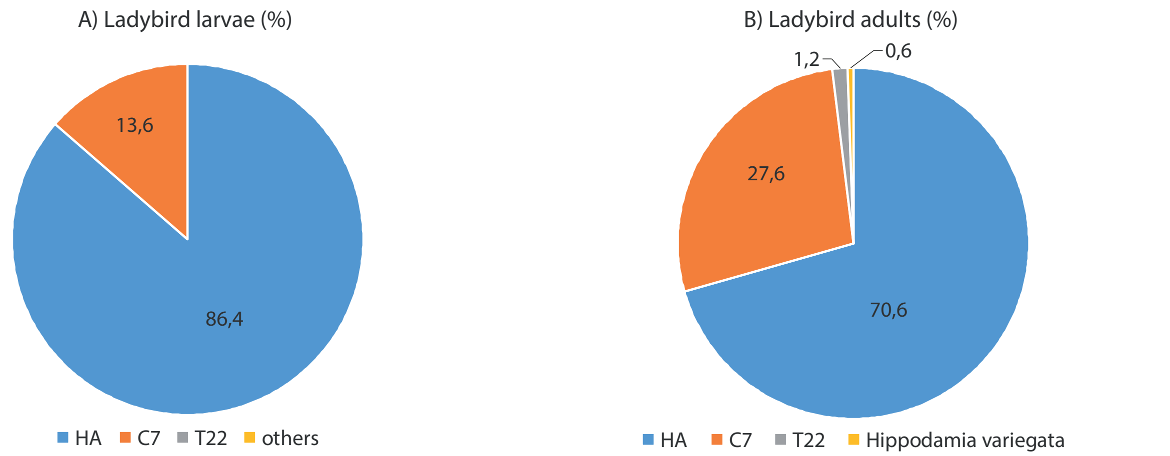

Fig. 4 Percentage representation of particular ladybird species in 2017, when trees were sampled: A) larvae, B) adults.

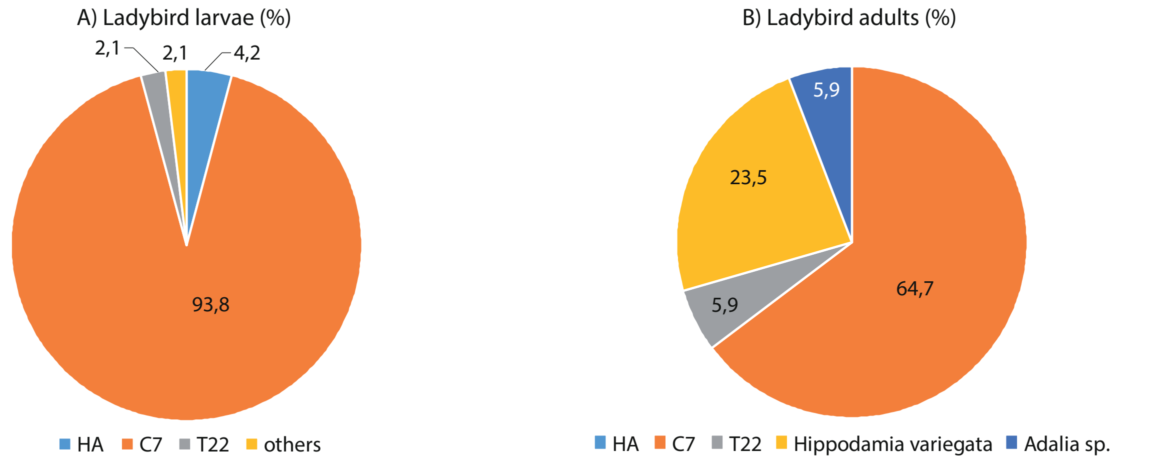

Fig. 5 Percentage representation of particular ladybird species in 2018, when trees were excluded from the sampling: A) larvae, B) adults.

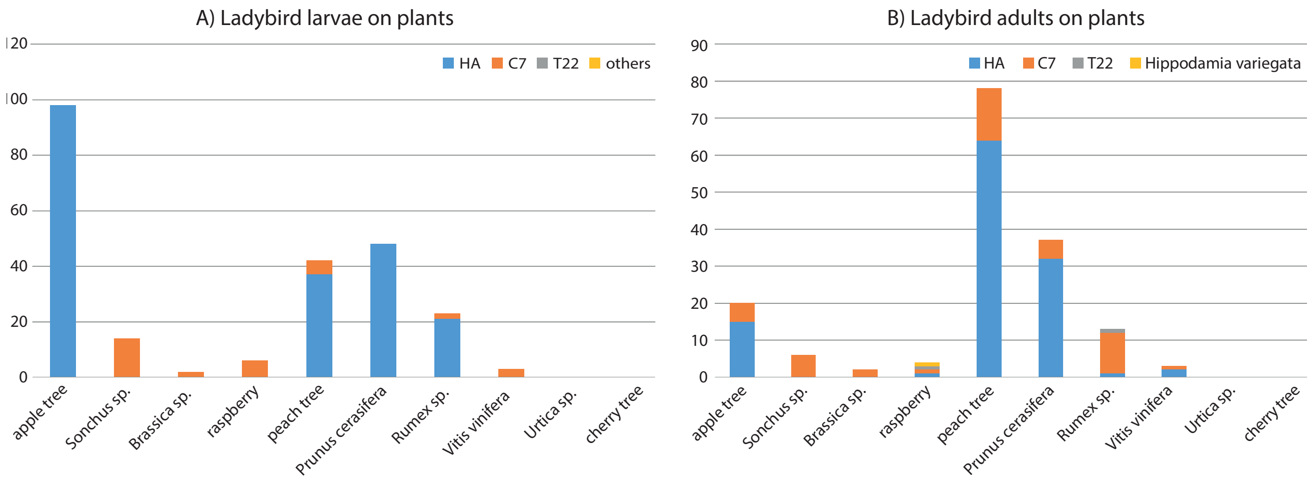

Fig. 6 Plants where ladybirds were observed in 2017: A) larvae, B) adults.

one of the reasons, why *Harmonia axyridis* is not very successful in the Mediterranean: the aphid colony may exist for a period of time too short in the Mediterranean conditions to enable successful development of *H. axyridis*. The results of this analysis are still under preparation, however.

Conclusions

When trees were not sampled, majority of ladybird adults and larvae was of *Harmonia axyridis* species, but on the other hand, when trees were excluded from the sampling, the dominating species was *Coccinella septem-*

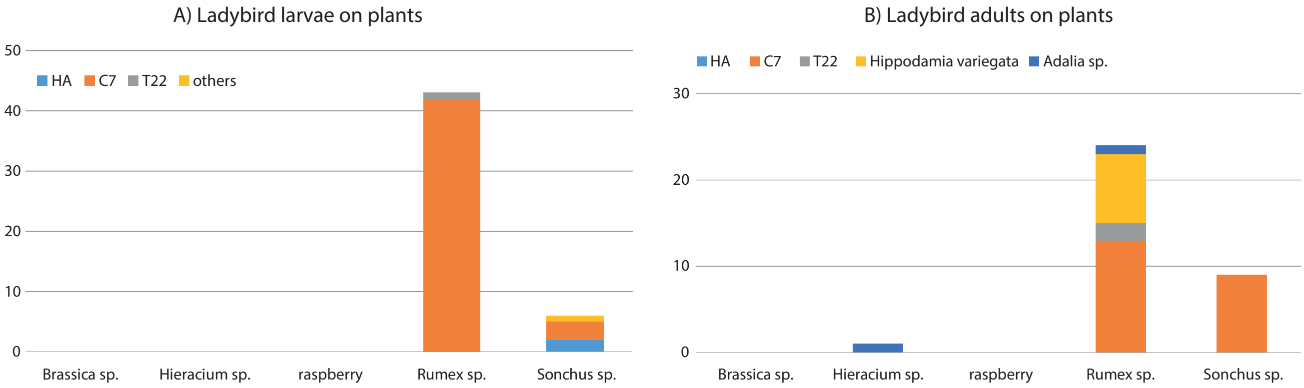

Fig. 7 Plants where ladybirds were observed in 2018: A) larvae, B) adults.

punctata. It may imply that in the Mediterranean climate, *H. axyridis* has a limited time to reproduce and create a new generation.

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REFERENCES

Brown PMJ, Frost R, Doberski J, Sparks T, Harrington R, Roy HE (2011) Decline in native ladybirds in response to the arrival of *Harmonia axyridis*: Early evidence from England. Ecol Entomol 36: 231–240.

Hilton-Taylor C (2000) 2000 IUCN red list of threatened species. IUCN.

Kindlmann P, Arditi R, Dixon AFG (2004) A simple aphid population model. In: Simon JC, Dedryver C-A, Rispe C, Hullé M (eds.) Aphids in a new millenium, INRA, Paris, France, pp. 325–330.

Kindlmann P, Dixon AFG (1999) Strategies of aphidophagous predators: lessons for modelling insect predator-prey dynamics. J Appl Entomol 123: 397–399.

Pervez, A, Singh PP, Bozdoğan H (2018) Ecological perspective of the diversity of functional responses. Eur J Environ Sci 8: 97–101, https://doi.org/10.14712/23361964.2018.13.

Pervez, A., Yadav, M.: Foraging behaviour of predaceous ladybird beetles: a review. Eur J Environ Sci 8: 102–108, https://doi.org/10.14712/23361964.2018.14.

Roy HE, Adriaens T, Isaac NJB, Kenis M, Onkelinx T, Martin GS, Brown PMJ, Hautier L, Poland R, Roy DB, Comont R, Eschen R, Frost R, Zindel R, Van Vlaenderen J, Nedved O, Ravn HP, Gregoire JC, de Biseau JC, Maes D (2012) Invasive alien predator causes rapid declines of native European ladybirds. Divers Distrib 18: 717–725.

Roy HE, Brown PMJ, Adriaens T, Berkvens N, Borges I, Clusella-Trullas S, Comont RF, De Clercq P, Eschen R, Estoup A, Evans EW, Facon B, Gardiner MM, Gil A, Grezz AA, Guillemaud T, Haelewaters D, Herz A, Honek A, Howe AG, Hui C, Hutchison WD, Kenis M, Koch RL, Kulfan J, Handley LL, Lombaert E, Loomans A, Losey J, Lukashuk AO, Maes D, Magro A, Murray KM, San Martin G, Martinkova Z, Minnaar IA, Nedved O, Orlova-Bienkowskaja MJ, Osawa N, Rabitsch W, Ravn HP, Rondoni G, Rorke SL, Ryndevich SK, Saethre MG, Sloggett TT, Soares AO, Stals R, Tinsley MC, Vandereycken A, van Wielink P, Viglasova S, Zach P, Zakharov IA, Zaviezo T, Zhao ZH (2016) The harlequin ladybird, *Harmonia axyridis*: global perspectives on invasion history and ecology. Biol Invasions 18: 997–1044.