https://doi.org/10.17221/142/2020-HORTSCI

Storage of proliferating gooseberry cultures under slow growth conditions

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Citation: Kucharska D., Orlikowska T., Maciorowski R., Kunka M., Niewiadomska-Wnuk A. (2021): Storage of proliferating gooseberry cultures under slow growth conditions. Hort. Sci. (Prague), 48: 134–140.

Abstract: Short storage of *in vitro* cultures under slow-growth conditions is included in the commercial large-scale micropropagation process. It is dictated by the organizational scheme that provides temporary stop multiplication of shoots for some months. To avoid subculturing to fresh media every 4 weeks, which is obligatory for gooseberry, they can be kept in conditions that protect them from ageing, by slowing down their metabolism. To develop a rational schedule of gooseberry micropropagation, two experiments were used to adopt a temperature and length of time for storage. The best results were obtained with storage conditions at 2 °C for two or four months for proliferating cultures. Under these conditions, the percentage of necrotic shoots was low (< 10%), and shoot proliferation in the subsequent passages was at a level similar to proliferation cultures incubated in the growth room and sub-cultured monthly. The rate of shoots > 1 cm was higher than in the control in the growth room. Storage at 4 °C increased the probability of necrotic shoots up to 80% and decreased the number of all shoots and shoots > 1 cm in subsequent passages.

Keywords: *Ribes grossularia* L.; micropropagation; necrotic explants; storage

Gooseberry has been cultivated for its fruit for a long time, but its cultivation began on a larger scale due to the development of cultivars that were better adapted for machine harvesting (James 1980; Pluta 2018). Gooseberry fruit is considered to have pro-health and low-caloric value. Poland is one of the largest gooseberry producers in Europe and the world (Pluta 2018).

Like other vegetatively propagated species, gooseberry plants transfer pathogens, especially viral ones, through planting materials. In addition, gooseberry has low rooting efficiency using traditional methods (Debnath 2003). These are reasons that *in vitro* cultures are being adapted for the production of virus-free planting material of gooseberry (Jones, Vine 1968). *In vitro* multiplication guarantees that if the donor plants are free from viruses and other pathogens, the microplants will also continue this sanitary status. In the absence of pathogen-free donor plants, virus-free material can be obtained by initiating cultures from meristems, especially those pretreated with a higher temperature (thermotherapy) (Wang, Hu 1980). Gooseberry is a species that is not easily adaptable to efficient micropropagation. Therefore, micropropagation is mostly limited to the production of virus-ree material that is used for planting in nurseries from which cuttings for traditional rooting are derived or for the quicker introduction of newly developed cultivars to fruit production.

Plants produced *in vitro* are juvenilized, which results in the ability for rapid growth, more dy-

Supported by the Research Institute of Horticulture, Poland, Statutory Found, Project No. 3.3.5.

https://doi.org/10.17221/142/2020-HORTSCI

namic biomass gain, and higher rooting efficiency than those produced traditionally through cuttings (Dubois et al. 1988; Howard et al. 1989; Drew, Smith 1990). In our field experiment, gooseberry genotypes produced *in vitro* had twice as much shoot growth and bushes that were three times wider, as well as half the percentage of fruiting plants in the second year of growth (Wójcik et al. 2020). This indicates that *in vitro* propagated plants of gooseberry are a proper source of cuttings, but in the first year of fruiting, they are less productive.

Although gooseberry can be multiplied *in vitro* throughout the year, their acclimatization and transfer to the field are most effective in early spring. For this organizational requirement, shoot cultures are being kept without sub-culturing onto fresh media under slow-growth conditions for a few summer and autumn months.

At the Research Institute of Horticulture, a breeding program aiming to develop new cultivars of gooseberry are underway, as well as experiments are performed on propagation and cultivation, and qualitative assessment of fruits (Pluta 2018). For the purposes of rapid multiplication of selected clones, we have developed an effective method of micropropagation using meta-topolin (m-T), instead of benzylaminopurine (BAP) (Kucharska et al. 2020). In the current work, we present the results of experiments carried out on the short-term storage of gooseberry culture at the multiplication stage in slow-growth conditions.

MATERIAL AND METHODS

The material for the experiments were multi-shoots of 10 gooseberry genotypes (3 cultivars and 7 breeding clones) that were in vital condition. They were incubated for 4 weeks on a shoot multiplication medium. Growth media contained salt and vitamin sets of Murashige and Skoog (Murashige, Skoog 1962), 100 mg/L inositol, 85.45 mg/L $MgSO_4$, 0.1mg/L gibberellic acid (GA_3), 0.1 mg/L indole 3-acetic acid (IAA), 0.5 mg/L *meta*-topolin (*m*T,) sucrose 30 g/L, and Bacto agar 7.0 g/L with a pH of 5.8. All nutrient components were purchased from Duchefa (the Netherlands), except Bacto agar that was produced by Benton Dickinson (USA).

The temperature in the growth room was 23–25 °C with about 55–65% air humidity and a 16 h photoperiod with light emitted by fluorescent lamps (Cool Day Lamps, Pila, Poland) and irradiation of 60 μmol/m^2/sec.

The cultures were placed in a cold room at 2 or 4 °C in the dark for 2, 4, or 6 months. After these periods, cultures were transferred to a growth room, and evaluation was made within 1–2 days (subculture 0). In each combination, there were ten jars with ten multi-shoots each. The evaluation included the number of all shoots that could be separated from the multi-shoot and the number of necrotic shoots. Necrotic shoots included shoots with dark discolouration and shoots with whitened leaves and without turgidity (dry). After evaluation, good quality shoots longer than 5 mm were transferred to a fresh proliferation medium. After four weeks, cultures were evaluated in a similar way as, directly after storage, except that the number of shoots ≥ 1 cm was also counted (subculture I). The evaluation also included subculture II to determine the after-effect of cold storage on the multiplication of shoots. In each treatment, there were 6 jars with 10 shoots each.

Two experiments were performed:
(1) Shoots were stored at 2 °C for 2, 4, or 6 months.
(2) Shoots were stored at 4 °C for 2, 4, or 6 months

The distribution of the examined traits was evaluated by the Kolmogorov-Smirnov test. Because the traits were non-normally distributed, generalized linear models (GLM) were used for data analyses. The number of shoots was analyzed by means of GLM with a logarithmic link function because they had a Poisson distribution. The occurrence of shoots greater than 1 cm and necrotic shoots were treated as binominal variables and modelled using GLM with the logistic link function. All models were built as a model with a baseline level, which was 2 °C, 2 months, and cultivar Hinsel. The Wald test was used to test the estimated main effects and their interactions. When the tested effect was significant, type I likelihood ratio statistics (LRT test) were used. Calculations and analyses were made using Statistica v. 13 (Dell Inc. 2016).

RESULTS

The appearance of cultures after storage depended primarily on the temperature. Cultures stored at a temperature of 2 °C had mostly green leaves, and those from a temperature of 4 °C had mostly white or yellow leaves (Figure 1). All factors, including storage temperature, time of storage, and genotype, influenced the survival of gooseberry cultures during incubation in slow growth conditions and during subsequent sub-cultures (Table 1). Less necrotic

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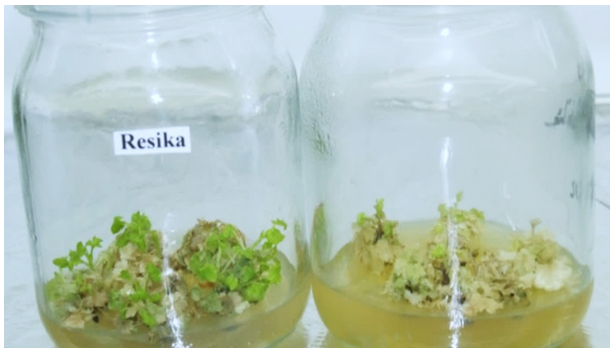

Figure 1. Comparison of the quality of gooseberry cv. 'Resika' *in vitro* after 6 months of storage at 2 °C (on the left) and 4 °C (on the right)

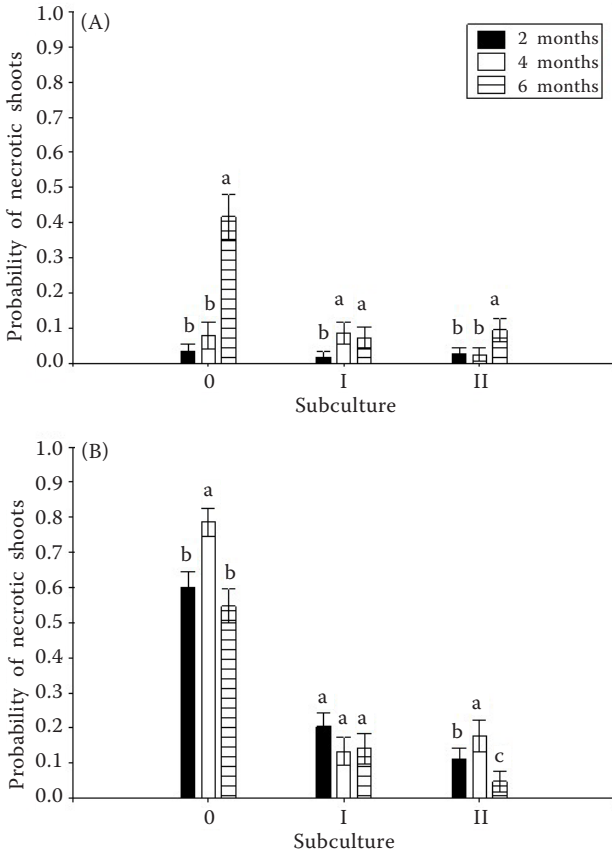

Figure 2. Probability of necrotic shoots of gooseberry, stored for 2, 4, or 6 months at 2 °C (A) and 4 °C (B)

Data are averages over all tested cultivars; the same letters above bars indicate that means do not differ significantly at $P = 0.05$, separately for each subculture according to the *LRT* test; the horizontal line represents the average of two passages of cultures incubated in the growth room

shoots were found in cultures incubated at 2 °C than at 4 °C (Figure 2). Incubation at 2 °C caused an increase in the probability of necrotic shoots close to zero after 2 months of storage and 0.44 after 6 months of storage. During the first and second subculture periods, the probability of necrotic shoots was lower than 0.1 (Figure 2A) and was about the same as between shoots not stored and those sub-cultured every 4 weeks. Incubation of cultures at 4 °C caused an essential increase of necrosis from 0.55 to 0.78 (Figure 2B). In the first subculture, this value was from 0.15 to 0.23, and in the second subculture, from 0.05 to 0.13.

The number of necrotic shoots was also genotype dependent (Figure 3). For individual genotypes, the average percentage of necrotic shoots stored at 2 °C differed from 0 to 16% after 2 months of storage, from 0 to 38% after 4 months, and from 2 to 73% after 6 months (Figure 3A). After storage at 4 °C, the percentage of necrotic shoots increased from 11% to 81% after 2 months, from 7% to 87%

Table 1. Probability of Wald test statistic for tested effects and their interaction in GLM models estimated for investigated characters

Effect	Subculture/trait								
	0			I			II		
	No. of shoots	probability of shoots > 1 cm	probability of necrotic shoots	No. of shoots	probability of shoots > 1 cm	probability of necrotic shoots	No. of shoots	probability of shoots > 1cm	probability of necrotic shoots
Temperature (T)	0.000	–	0.000	0.000	0.112	0.000	0.019	0.055	0.000
Cooling time (Co)	0.000	0.000	0.000	0.003	0.006	0.787	0.000	0.000	0.130
Cultivar (Cu)	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.000
T × Co	0.000	–	0.000	0.000	0.520	0.000	0.000	0.000	0.000
T × Cu	0.004	–	0.000	0.000	0.000	0.000	0.001	0.000	0.000
Co × Cu	0.048	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
T × Co × Cu	0.003	–	0.000	0.000	0.000	0.000	0.000	0.000	0.000

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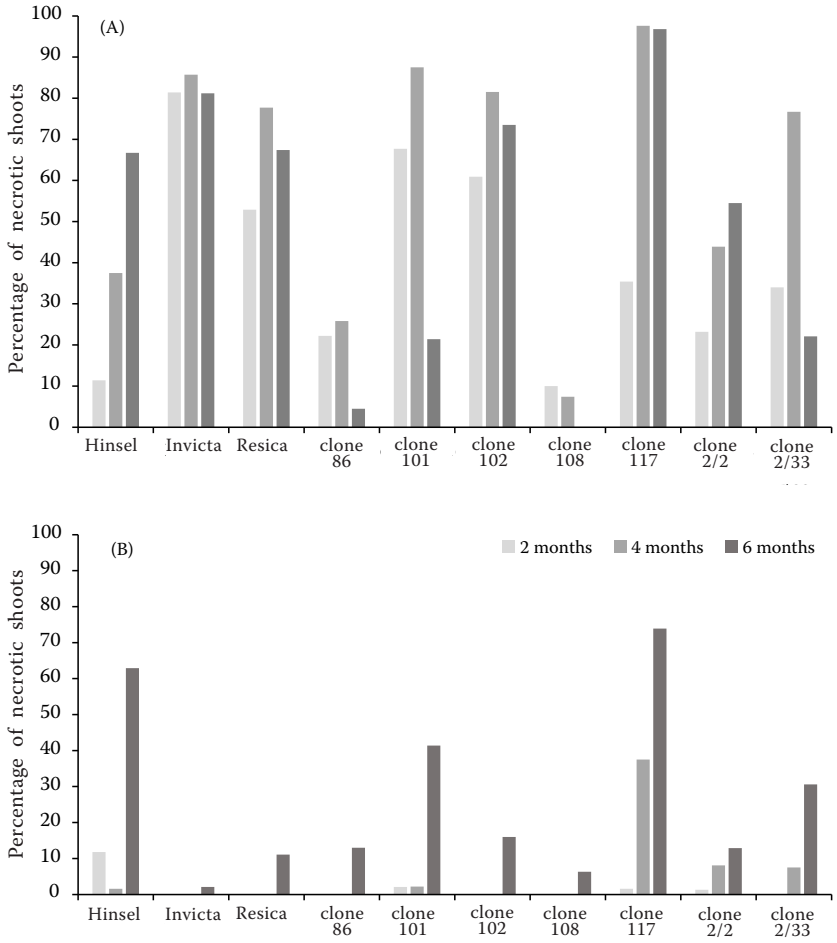

Figure 3. Percentage of necrotic shoots of gooseberry in different genotypes per explant stored at 2 °C (A) and 4 °C (B) after different storage durations

after 4 months, and from 0 to 97% after 6 months (Figure 3b). The most susceptible genotypes were 'Hinsel' and clones 101 and 117 at 2 °C and 'Invicta', 'Resica' and clones 101 and 102 at 4 °C.

The number of all shoots present at the time of removal from cold storage depended on temperature. It was higher in cultures stored at 4 °C and decreased with storage time, but in cultures stored at 2 °C, it increased with storage time. (Figure 4). The shoot number in subcultures I and II were relatively similar or higher among cultures stored at 2 °C. A higher number of shoots was found in cultures stored for two and four months. Cultures stored at 4 °C proliferated worse in subsequent subcultures, especially in the first subculture. In the first subculture, shoot number was not dependent on storage time at 4 °C. In the second subculture, proliferation was higher at both storage temperatures. The number of shoots > 0.5 cm was generally much lower than in cultures that were sub-cultured every 4 weeks and incubated in the growth room. The decrease in shoot number was especially high in cultures originating from those stored at 4 °C.

In subcultures I and II, a probability of shoots > 1 cm was generally higher in cultures stored at 2 °C than at 4 °C and compared with cultures that were not stored. More shoots > 1 cm occurred in the second sub-culture than in the first under both temperature regimes (Figure 5). In the first and second subculture, the highest number of long shoots were scored among cultures stored for 4 months. The frequency of shoots > 1 cm between cultures stored at 4 °C was higher when shoots were stored for 4 or 6 months than for 2 months. Generally, storage at 2 °C and 4 °C favoured further shoot elongation.

DISCUSSION

To our knowledge, the first person that attempted to conserve plant tissue cultures was Caplin (1959). He applied a method used in microbiology, namely, the overlay of *in vitro* explants with mineral oil. His

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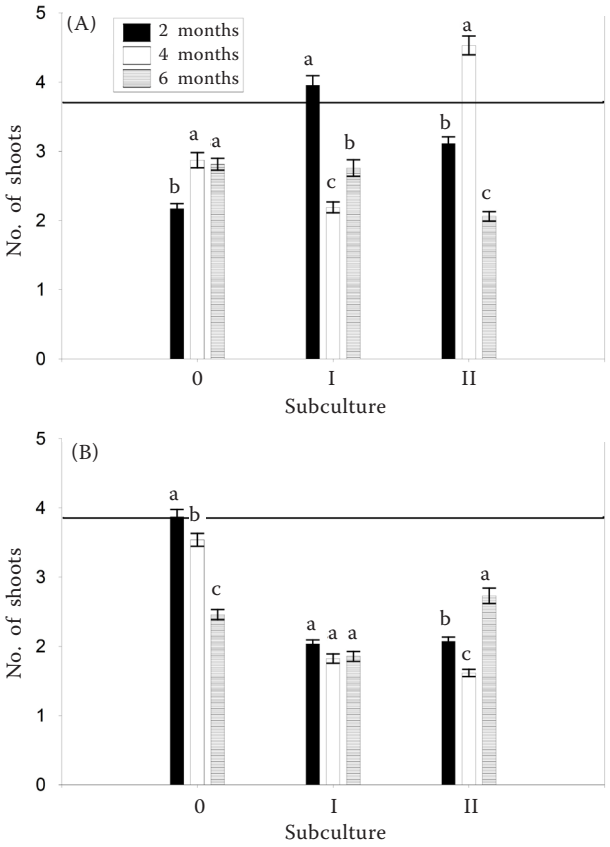

Figure 4. Number of shoots of gooseberry stored for 2, 4, or 6 months at 2 °C (A) and 4 °C (B)

Data are averages over all tested cultivars; the same letters above bars indicate that means do not differ significantly at $P = 0.05$, separately for each subculture according to the *LRT* test; the horizontal line represents the average of two passages of cultures incubated in the growth room

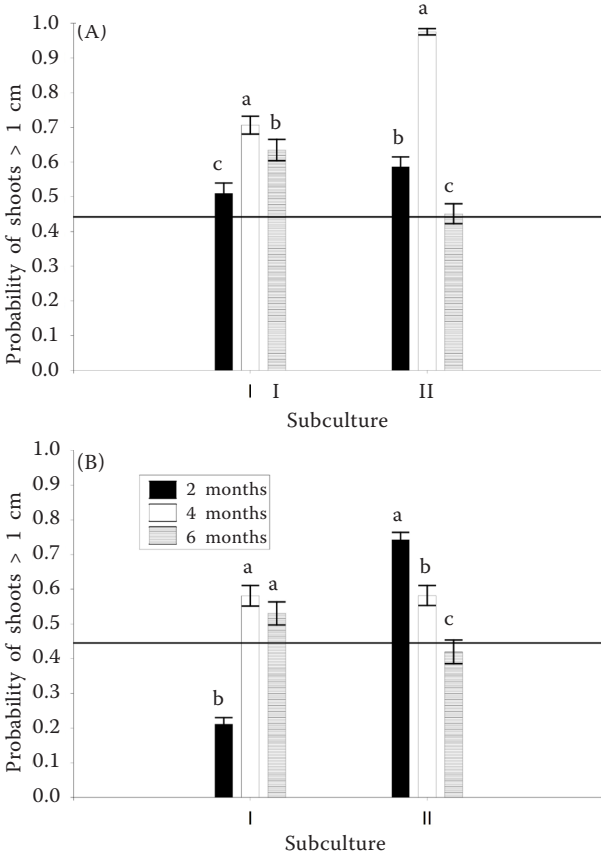

Figure 5. Probability of gooseberry shoots > 1 cm stored for 2, 4, or 6 months at 2 °C (A) and 4 °C (B)

Data are averages over all tested cultivars; the same letters above bars indicate means that do not differ significantly at $P = 0.05$, separately for each subculture according to the *LRT* test; the horizontal line represents the average of two passages of cultures incubated in the growth room

idea is continued recently by de Lacerda et al. (2020) who used conservation under mineral oil together with low temperature (15 °C) for storage of tropical plants (*Pfaffa glomerata* and *Lippia filifolia*). Strawberry has long been micropropagated for commercial purposes, and the a method of strawberry cultures storage was adapted for industrial scale micropropagation and gene banks. Mullin and Schlegel (1976) reported the possibility to store 50 *Fragaria* clones at 4 °C in the dark for 6 years with regular replenishment of liquid medium. Boxus and Druart (after Aitken-Christie, Singh 1987) were able to store 200 *Fragaria* genotypes at 4 °C for 10 years. Presently, many protocols and scientific conclusions have been made on *in vitro* storage.

The possibility to safely keep living tissue cultures in a state of low metabolism depends on several factors, including temperature, medium composition, light intensity, type of vessels, and period of storage (Aitken-Christie, Singh 1987; Engelman 2011). The most important is low temperature, which differs according to species. Plants of the temperate zone are usually maintained at –1 to 5 °C, whereas those that are cold-sensitive at 10 to 25 °C (Cha-um, Kirdmanee 2007). Different species respond differently to storage conditions; some increase their multiplication potential as a result of storage, producing more and longer shoots, but some decrease the ability to proliferate and elongate shoots. This can be partly connected to endodormancy (Borkowska 1986).

Margareta Welander (Welander 1995) was the first to report the results of experiments on the storage of gooseberry shoot clusters cv. 'Hinnomaki Yellow' on Lepoivre medium without growth regulators. Her

https://doi.org/10.17221/142/2020-HORTSCI

experiment included storage temperature (5 °C or 10 °C), darkness or low light, immediate or gradual transfer to a growth room, and a period of storage (73 or 130 days). All cultures survived storage for 73 days but after 130 days, some cultures died. However, all cultures survived this period in conditions of low light, 10 °C, and gradual transfer to a growth room at temperatures 24 °C through 16 °C. Multiplication after storage was not studied. Gunning and Lagerstedt (1985) reported inconclusive and mixed results on the storage of *Ribes* genotypes, indicating that most genotypes survived one year of storage at –1 °C. Brennan et al. (1990) stored three cultivars of *Ribes* at 6 °C for three months with good survival and morphogenic potential. Reed and Chang (1997) (after Reed, Hammer 2002) stored 80 *Ribes* genotypes at 4 °C for 1.4 years under a 12 h photoperiod and 40 accessions were able to survive 2.76 years at –1 °C. Thereafter, Reed and Hummer (2002) developed a protocol for the storage of *Ribes* spp., including gooseberry, which was usable for both cold storage of shoots *in vitro* and cryopreservation of meristems. In their protocol, three important factors were indicated: (a) storage medium, which was MS salts and vitamins with ammonium and potassium nitrate reduced to 30%, plus 50 mg/L ascorbic acid, 20 g/L glucose, 3.5 g/L Bacto agar, and 1.75 g/L Gelrite; (b) polyethylene bags containing one shoot each were used instead of glass vessels; and (c) cold acclimatization through gradual temperature lowering, up to –1 °C. Shoots were evaluated with a 0–5 vigour scale at 3–4 month intervals.

When drawing up a micro-propagation scheme that considers the period without transplantation into fresh media, it is important to predict the survival rate, as well as the proliferation efficiency after a specified period of storage under certain conditions. In our experiments, survival was strongly connected with temperature and storage length. At 4 °C, more necrotic shoots occurred when compared with the same storage time at 2 °C. This effect was continued in subcultures I and II. A temperature of 4 °C also negatively affected the efficiency of shoot formation in subsequent subcultures. The number of shoots was reduced by half of those in cultures incubated in the growth room. Additionally, the number of shoots > 1 cm was higher after storage at 2 °C than at 4 °C.

Lisek and Orlikowska (2008) stated that the ability of strawberry cultures to produce axillary shoots decreased with the duration of storage, more so in the first sub-culture than in the second compared with control cultures sub-cultured every 5 weeks. The typical proliferation potential recovered in the third subculture.

The obtained results on the storage of gooseberry cultures indicate that better survival and further proliferation was characteristic for cultures stored at 2 °C for 2 or 4 months.

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Received: October 2, 2020
Accepted: January 26, 2021
Published online: September 21, 2021