Thesis of Doctoral (Ph.D.) Dissertation

UTILIZATION OF SUPPLEMENTAL SHRUB MALLOW SPECIES IN THE TEMPERATE ZONE

Erika Kurucz

Supervisor:

Prof. Dr. Miklós Gábor Fári

university professor

UNIVERSITY OF DEBRECEN

Kerpely Kálmán Doctoral School

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1. INTRODUCTION AND AIMS

A modern approach of landscape architecture would be to use new ornamental plant varieties which can tolerate the urban and extensive circumstances, while their biomass production ability and by-product utilization potential could contribute to the multi-purpose utilization of the landscape; i.e., to the sustainable and healthy development of urban regions. This direction of ornamental and biomass plant breeding was represented by Dr. Zoltán Kováts in Hungary, who increased the value of this field to international level from 1960's to 2010, the year of his passing. According to the renowned researcher, the complex and promising solution of the above specified problem is to initiate the utilization of shrub or semi-shrub mallow taxons from the temperate zone. There was an exemplary professional collaboration between Prof. János Domonkos and Dr. Zoltán Kováts and continued in the fifties of the last century (Honfi - Steiner, 2013). The establishment of modern ornamental plant breeding based on this knowledge should be a valuable opportunity of ornamental plant production, also considering climate change, as well as other old and new challenges (Domokos, 1934, 1957; Kováts, Gracza, et al., 1978; Kováts, Mustafa, et al., 1978; Kováts, 1980). The dual direction determined by Zoltán Kováts 30-60 years ago is innovative and sustainable even nowadays regarding many species. There are numerous examples in the field of ornamental and practical utilization of mallow species, such as the ornamental and dyeing mallow's breeding programme and the biotechnology assisted biogeneration plant research within the giant mallow species (*Kitaibela vitifolia* Willd., *Napea dioica*). The breeding of these species tends to create an opportunity of biomass production under a marginal condition and to have decorative value in an urban environment (Fári et al., 2014). The establishment of the "Háros" genus hybrid and the research of *Kitaibela vitifolia* Willd and *Kitaibela balansae* Boiss, as well as their hybrids and the study of *Sida hermaphrodita* were motivated by the same reason.

The aim of my research is to study these mallow species from the aspect of multi-purpose utilization, continuing the innovative research work originally started by Kováts Zoltán and the legal predecessor of the Faculty of Agricultural and Food Sciences and Environmental Management of the University of Debrecen.

2. MATERIAL AND METHODS

2.1. Morphological characterization of the *Kitaibela vitifolia* Willd. x *Kitaibela balansae* Boiss hybrid (Kvb hybrid) population

The examined population was gained from the plantlet originated from F1 seeds of the self-fertilized population of KVB hybrid plants in 2011 in the Demonstration Garden of the University of Debrecen (47°33'00.4"N 21°36'02.0"E) The plantlets were directly transplanted in the grass, applying a shallow planting pit. Morphologic observations were carried out in 2014, and the following characters were examined: pink flower color (1), dropping of the destructive, browned inflorescences after blooming (2), and other two aesthetically important traits, the habus(3) and the leaves' shape(4).

2.2. Plant material of the photosynthetic investigation

During the determination of certain photosynthetic parameters, mainly the yellow-leaved variety was used, which was previously selected before on its temporary yellow leaves (at the beginning of the vegetation period). The *Kitaibela vitifolia* and the other selected plants (Kvb 1/25; 2/7) were considered to be control plants.

2.3. Determination of the chlorophyll content using the spectrophotometric method

The determination of chlorophyll and carotin content was performed based on the modified method of (Porra et al., 1989). A 10 mm diameter leaf disc was cut from fully developed leaves (3th-4th level). Chlorophyll fluorescence induction method was used during the photosynthetic activity characterization (Schreiber et al., 1995). The parameters of the fast stage of *in vivo* chlorophyll fluorescence induction were detected with a PAM-2001 type flurometer (WALZ Gmbh, Germany).

2.4. Examination of the semi shrub mallow species and interspecific and intergenomic hybrids using the flow cytometry method.

The plant materials of the cytological examinations were collected on five occasions (2015.05.21; 2015.05.27; 2015.05.28; 2015.06.06, 2015.07.01) in the Demonstration Garden of the University of Debrecen. Fresh leaves and meristematic tissue (mainly shoot tip) were used in the examination. Collection and preparation of *Bellis perennis* in the same manner and at the same time was the internal standard during the C-value determination. Sample preparation

was carried on based on the (Hsiao-Ching - Tsai-Yun, 2005) technique with some modification. The collected samples were measured within 20 minutes after preparation. The obtained data were analyzed using the Flowing software (http://www.flowingsoftware.com.). The relative DNA content was calculated using the following formula: 2C DNA content of the sample = (median of the sample G1 peak/ median of the standard G1 peak)/ 2 C content of the standard in pg (Dolezel - Bartos, 2005).

2.5. Examination of the genetic diversity of the mallows interspecific and intergenomic hybrids using molecular genetic markers

The genomic DNA was isolated with the Maceny Nagel Plant II DNA isolation kit (IZINTA Ltd.) based on the official protocol provided by the manufacturer. The following primers were applied: RAPD: OPI-10,OPI-05, OPZ-04,OPZ-10, SSR: UBC 818, UBC 807, UBC 810, UBC-841,UBC-856. The gained PCR products were separated with gel electrophoresis, in 1.5% agarose gel. The gel was visualized in a UV-transilluminator and photographed in the UVIpro Platinum Gel documentation System (camera: 1.1 mega-pixels, 1184x890) and analyzed with PhlxElph 2.5.6. software. The genetic distance was calculated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

2.6. Seed biology assay of examined mallows species and interspecific hybrids

2.6.1. Plant material

The mother plants of *Sida hermaphrodita*, *Althea cannabina* and *Kitaibelia vitifolia* were planted in 2009-2010 in the biomass plots of the Demonstration Garden of the University of Debrecen.

2.6.2. Seed composition assays

The mallow seeds composition assays were carried out in the laboratory of BUNGE Ltd, in Martfű by Éva Nagy, Tiborné Nagy and József Kucsa (head of laboratory) using GC MS and in accordance with the MSZ ISO standard no. 659:1990 (659:2009, 2010).

2.6.3. Germination conditions

The germination tests were performed after surface washing with distilled water in autoclave sterilized Petri-dishes with a sterile filter paper. Every treatment was replicated four times using

50 seeds in each replication, in March 2013 (*Althea cannabina*, *Kitaibela vitifolia)* and in March and April 2014 in the case of *Sida hermaphrodita.* In the case of *K. vitifolia*, the cleaned seeds were kept at 4 ^{o}C (cold pretreatment) for two days. The hot water pre-treatment was executed in a 80 ^{o}C bath for two minutes in the case of *Sida hermaphrodita*. The germination assay was carried out in a sterile Petri dish with sterile filter paper. Due to the limited amount of *Althea cannabina* seeds, only the seed fractionating assays could be carried out. Seed fractionation tests were performed in the same manner for all species, i.e., the seeds were put in distilled water for 30 minutes, and the separation was done based on their density and/or imbibation ability, separating the flowtrough and the shrink seeds.

2.7. In vitro propagation of the horticulturally valuable *Kitatibelia* interspecific hybrid (Kvb) genotype

The plant material originated from the interspecific hybrid populaton of *Kitaibelia vitifolia X K. balansae* in the Demonstration Garden of the University of Debrecen. The mother plant was the genotype with pink petals (Kvb-1-1-1). This genotype was infected and destroyed by white mold (*Sclerotinia sclerotiorum*) in 2015.

2.7.1. Culture condition

The starter media was supplemented with MS-salts including vitamin complex (Murashige - Skoog, 1962), 4.4 g l^{-1}, 3% saccharose, 0.1 g l^{-1} Schenk and Hildebrandt Vitamin (Sigma-Aldrich, hereinafter referred to as vitamin S and H) and additionally 0.2 mg l^{-1} α-naftilaceticacid (Sigma-Aldrich, hereinafter referred to as NAA), as well as 0.2 mg l^{-1} 6-Furfurylaminopurine (Sigma-Aldrich, hereinafter referred to as KIN).

2.7.2. Evaluation of in vitro callus and shoot development influenced by NAA and KIN

0.6-1 g fresh weight callus of Kvb explants developed in the starter media were transplanted into five different types of induction media in 100 ml Erlenmeyer flask in 3 replications. The composition of the media is shown in Table 1. The BAP strong callus induction ability was observed by Szarvas et al. (2008), which was replaced by KIN. Kin has the same callus induction capacity using a high dosage in the case of *Alcae* and *Althea* species. (Szarvas et al., 2008). Cultures were maintained in the culture room at 25 ± 1°C, under a 16-hour-long photoperiod using cool white fluorescent light (48μmol.m-2.s-1) for 30 days. The results were expressed using Callus index (Khosh-Khui - Sink, 1982), a number of microshoots per explant

and the degree of rooting. In the case of callus formation, the callus type and the frequency were determined after 30 days of incubation under the same circumstances.

$$\text{Callus index} = \frac{100 \text{ n x G}}{\text{N}}$$

n: the number of explants which produced calli

G: visual callus rate from 1 to 4, where 1 means the smaller size and 4 means the largest size of the callus

N-the number of all explants.

Table 1. The media composition for mallows callus and shoot

Code	Salt vitamin (4.4 g l^{-1})	carbohydrate (v/w%)	Gelling agent (l^{-1})	Another supplement (l^{-1})	NAA	KIN	pH
1.	MS	Saccharose (3%)	Gelright (2.2 g)	Vitamins S and H (0.1 g)	0.2	-	5.8
2.	MS	Saccharose (3%)	Gelright (2.2 g)	Vitamins S and H (0.1 g)	0.5	-	5.8
3.	MS	Saccharose (3%)	Gelright (2.2 g)	Vitamins S and H (0.1 g)	0.2	0.3	5.8
4.	MS	Saccharose (3%)	Gelright (2.2 g)	Vitamins S and H (0.1 g)	0.5	0.3	5.8
5.	MS	Saccharose (3%)	Gelright (2.2 g)	Vitamins S and H (0.1 g)	-	-	5.8

2.7.3. Morphogenic callus regeneration of the interspecific hybrid of Kitaibelia Kvb

The organogenic callus clusters originated on the media type 4 were explanted (0.1-0.2 g each) into Petri dishes which contained different culture media supplemented with different type and concentration carbohydrate and salt source. The exact composition of the media was shown in Table 2. Based on our preliminary data, reduced sugar content (1, 1.5 %) was applied in order to avoid hyperhydrification. In addition, the salt concentration was also reduced in some cases (1/2 MS: 2.2 g l^{-1} MS complex). Data collection was performed after 20 days of the explantation by means of determining the number of shoots per cluster and calculating the rooting percentage.

$$Rooting\ \% = \frac{\text{rooted clusters}}{\text{total number of clusters}} \text{ x } 100$$

Table 2. The composition of the regeneration media of interspecific Kitaibelia hybrid.

Salts	Carbohydrates (v/w%)	Gelling agent (l^{-1})	Other compounds (l^{-1})	NAA	pH
MS	Saccharose (1%) wheat starch (2%)	Gelrite (1.1 g)	Vitamins S and H (0.1 g)	-	5.8
½ MS	Saccharose (1%) wheat starch (2%)	Gelrite (1.1 g)	Vitamins S and H (0.1 g)	-	5.8
MS	Saccharose (1%)	Gelrite (2.2 g)	Vitamins S and H (0.1 g)	-	5.8
½ MS	Saccharose (1%)	Gelrite (2.2 g)	Vitamins S and H (0.1 g)	-	5.8
MS	Saccharose (1.5%)	Gelrite (2.2 g)	Vitamins S and H (0.1 g)	0.1	5.8
MS	Glucose (1.5%)	Gelrite (2.2 g)	Vitamins S and H (0.1 g)	0.1	5.8
½ MS	Saccharose (1.5%)	Gelrite (2.2 g)	Vitamins S and H (0.1 g), KNO_3 (1 g)	-	5.8

Note: Based on some studies, in the case of the Malvaceae family, it should be more suitable to substitute partially or entirely the ammonium nitrate for the potassium nitrate for N force Sakhanokho et al. (2004) In consideration of these data, the last media was supplemented with 1g l^{-1} KNO_3 .

2.8. Vegetative propagation by means of shoot cuttings of the horticulturally valuable Kvb interspecific hybrid

A breeding stock of the selected genotype was established by means of shoot cuttings from young branches on 07/09/2017 with the collaboration of horticulturists Anikó Zsiláné André and László Zsila in their glass house and nursery. (H-4225 Debrecen-Józsa, Elek street 176). The starting material was the single nodal shoot segments of the selected individuals. The rooting and growing media was the premium quality "Jó Föld" seedling medium (phosphorus (P+) overdose, Pax 96 Kft., Kecskemét).

2.8.1. The study of seed biology and seed treatment efficiency of Sida hermaphrodita

The seeds originated from the population established in 2010. Germination tests were carried out in 2014 and seed samples were collected in 2011 (3 years old), 2012 (2 years old) and 2013 (1 year old) in early spring manually. Owing to the limited amount of seeds collected in the spring of the 2012 season, it was necessary to complement the samples with the seeds collected and cleaned after biomass collection.

Two-step seed treatment with water fractionation (HWT)

Based on the previous seed biology assay, the most effective temperature of 80°C was used for the seeds priming during the improved two-step pretreatment. Before the hot water treatment, the seeds were separated in distilled water for 30 minutes based on their imbibation capacity. The hot water treatment was performed with the imbibed/sunk seeds fraction. The seed priming tests were evaluated in the seeds of the 2012 season, collected after biomass harvesting.

2.8.2. Plantlet production experiences

Plantlet production at the end of summer

During the plantlet production experiences, we used nurse-in-tray technology, which consists of using single-space units (595x300x65) mm with seed densities of 30-40, and 50 per tray (0.18 m^2) Two seeds were placed in the previously established planting holes, each density in 4 replications. A thin layer of pine bark mulch was spread under the soil mixture. Also, a 60 x 60 cm foil was placed between the soil mix and the tray in order to remove the soil mixture interwoven with roots in one unit at the time of transplanting. The experiment was evaluated in the summer of 2013 (07). The germination percentage was recorded three weeks after sowing, taking double sown seeds into consideration.

2.9. Biomass yield production of *Sida hermaphrodita* Rusby

The University of Debrecen provided a 10 000 m^2 demonstration garden, half of which was used as mallow and other herbaceous biomass crops and/or ornamental plants, as well as half-wild and field crops used for various purposes. 5m wide and 45m long cultivated strips were established on the plot with similar grass-covered strips in between. According to the preliminary plans, 7 cultivated and 7 grassy blocks were established. During planting, three relatively wide spacings were used: 1m x 1m, 1m x 0.75m, and 1m x 0.5m. Harvesting was performed with hand tools. The data of inner stems were used during biomass yield calculation in the year between the seasons of 2012-2015.

2.10. Statistical analysis

All data are represented as mean ± SE, Descriptive statistical methods (sum, mean, standard deviation) and one-way ANOVA were used to determine the impact of treatments. Data were evaluated with Microsoft Excel and SPSS 11.0. The significant differences between each treatment were determined with Tukey's test at the probability level of 5%.

3. RESULTS

3.1. Morphological observations on the segregated interspecific hybrid populations of Kvb

As a result of the morphological observation of leaves, the following leaf types were identified:
type 1 (vitifolia leaves): The leaves are simple palmately veined with 5 lobes and the margins are dentate. The leaf base is heart-shaped and it has a light green color.
type 2 (with right angle base): The leaves are simple palmately veined with 5 lobes and acuminate tips and the margins are dentate. The leaf base is perpendicular to the petiole and its color is slate green.
type 3: The leaves are simple palmately veined with 3 lobes and acuminate tips, its margins are dentate, their colour is dark green and the base is heart-shaped
type 4 (balansae): The simple leaves have a heart shape, their margins are crenate and their colour was light green.

Concerning the morphology parameters, the population showed high diversity. As regards the yellow-leaved variety (marked with A) and the variety with pink petals, the height parameters were left behind compared to the *K. vitifolia* or other hybrid Kvb plants. however, some hybrids have higher biomass production capacity than the parent species. This phenomenon is in relation to the plants' variable growth habit (*Figure 1, Table 3*). The typical growth habit of *K.vitifolia* is strongly upright, while in the case of K. balansae all 3 types, i.e., upright, spreading and semi-spreading habits can be found. The traits valuable from the horticultural point of view, such as self-cleaning from overbloomed flowers or fruits and the pink color of the petals can be found within the population. Within the selected genotype, only one has self-cleaning ability, which increases the genotype's ornamental value. The color of the petals of the 'pink' progeny had different intensity in the flowers, from the sallow pink to fairly powerful pink petals. The genotype of the Kvb-2-4 had the most powerful petal color within the population. The spreading genotypes have a great value within the ground-cover species, due to the tolerance/adaptation capacity to extensive conditions. The new emerged shoots of these genotypes can cover the overbloomed fruits, thereby increasing its decoration value. In addition, this genotype bloomed for a longer time compared to the other semi-spread and erected progenies in the population (*1. figure*).

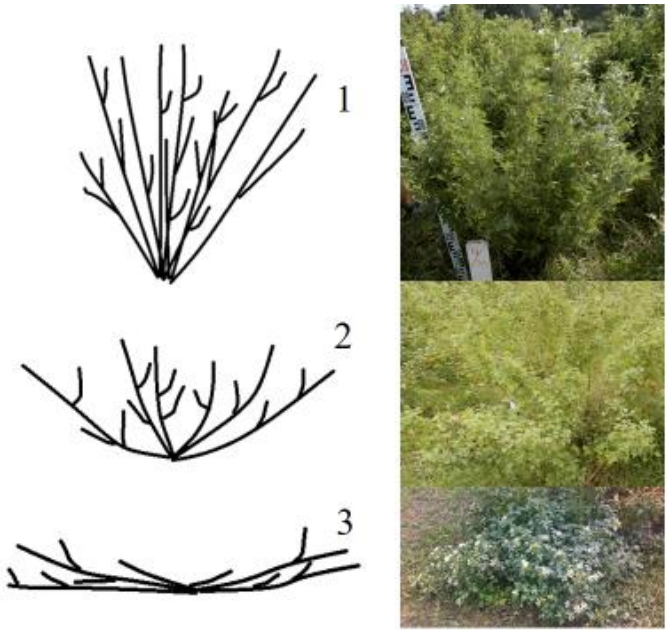

Figure 1. Kvb hybrids' typical growth habit

Table 1. Main characteristics of the individuals of hybrids *Kitaibela vitifolia* Willd x *Kitaibela balansae* Boiss emphasized from the horticultural aspect

Label	Habit	Leaf type	Petals colour	Fruit type	stem diameter (cm)	Plant height (cm)
Kitaibela vitifolia*	1	1	white	remaining	90	150-200 (measured 143.2 cm)
Kitaibela balansae*	1-3	4	pinkish -white	remaining	50	70-100
Kvb-6/19	2	1	white	self-cleaning	80	189
Kvb-2/7	3	2	pinkish- white	remaining	20-30	52
Kvb-1/26	1	2	pink	remaining	70	153
Kvb-2/4	2	3	pink	remaining	40	65
Kvb-1/10	2	4	pink	remaining	20	75
Kvb-A1	1	2	white	remaining	25	105
Kvb-A2	1	3	white	remaining	40	120
Kvb-A3	2	1	pinkish- white	remaining	45	115
Kvb-A4	1	3	white	remaining	60	119
Kvb-A5	2	1	white	remaining	20	42
Kvb-A6	1	1	white	remaining	20	83

Legends: Kvb –Kitaibela vitifolia x balansae 'kovatsii' hybrid serial number/ column number
A-aurea, yellow-leaved genotype, *literature source

3.2. Photosynthetic pigment content and assimilation efficiency of the yellow-leaved variety (Kvb-A) of *Kitaibela vitifolia* Willd x *Kitaibela balansae* Boiss hybrids

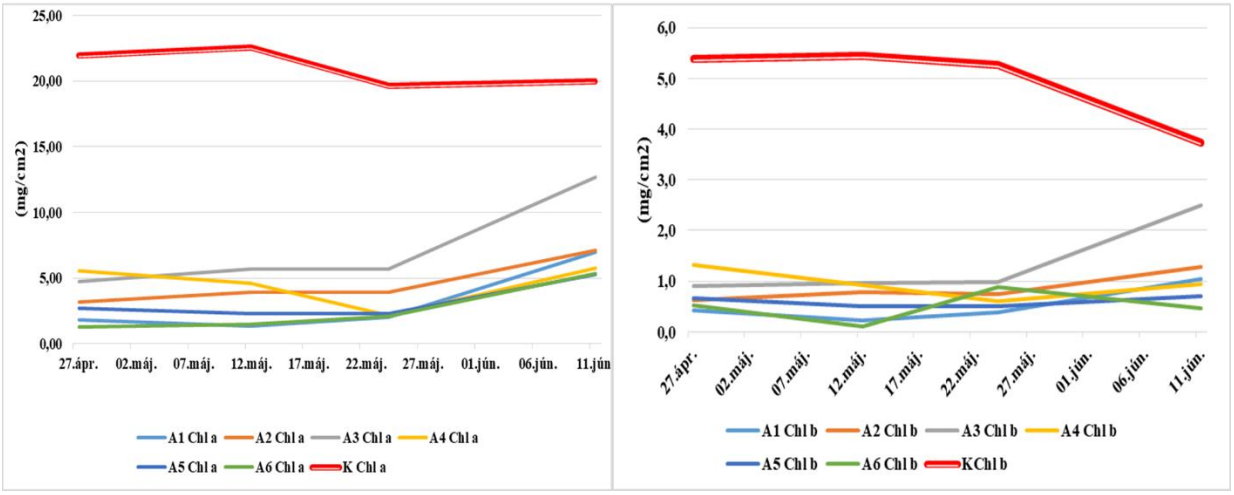

Figure 1. Photosynthetic pigment content of the Kvb hybrids, left: chlorophyll a, right: chlorophyll-b .

We carried out the observations on six selected yellow-leaved genotypes of *K. vitifolia* and *K. balansae* interspecific hybrids, which have such leaf color at the beginning of the vegetation period. The yellow varieties had much lower photosynthetic pigment content concerning both Chlorophyll-a and –b, and carotinoids in comparison with the control (1/26 variety). The lowest chlorophyll –a content was measured in the A6 genotype, the estimative chl-a content was 1.3 µg/ cm^2 , while the chl-a content was 20.1 µg/ $cm^{2.}$ in the case of the control (*Figure 2/left)*. The A3 genotype had the highest chl-a content within the yellow variety, amounting to 4.7 µg/ cm^2 in May and 12.7 µg/ cm^2 in June. The variety of A2, A3 and A4 gain their normal green color relatively early, but the A1 and A6 genotype remain yellow for a long period. The carotinoid and chlorophyll-b content show the same tendency as the chl-a (*Figure 2/right*). During the vegetation period, the amount of photosynthetic pigments increased considerably, which shows the metamorphosis of the leaf color.

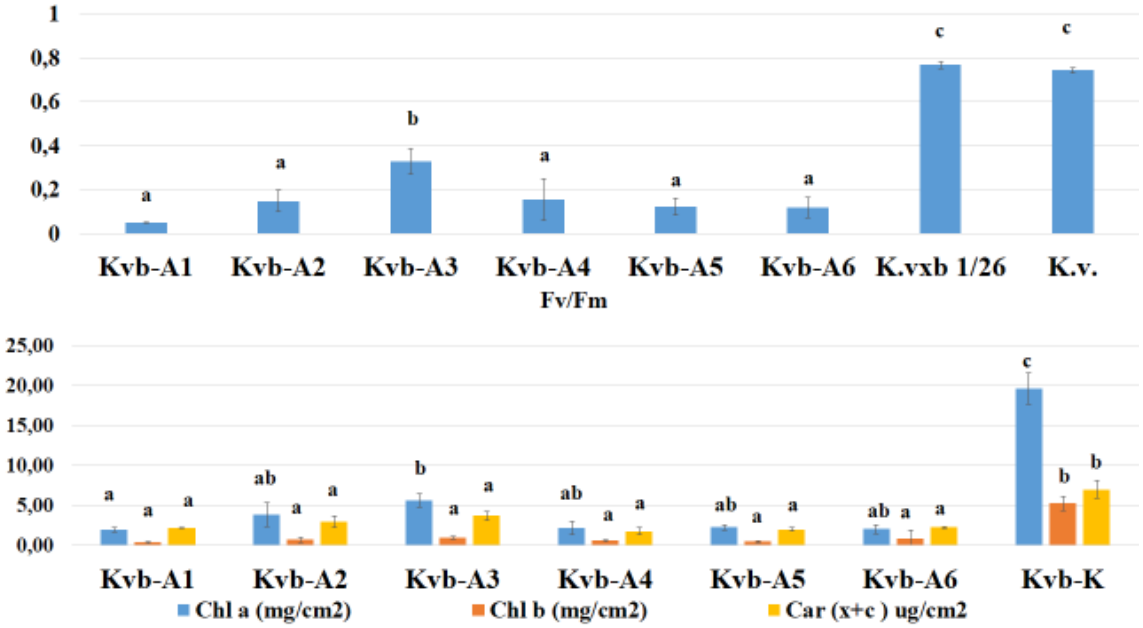

Figure 2. The Fv/Fm value and the actual photosynthetic pigment content of selected Kvb aurea genotypes (Debrecen, 2016)

Legend: Different letters show significant differences between the measured characteristics at the level of $\alpha = 0.05$ based on the TUKEY test.

The photosystem II efficiency of the yellow genotypes was markedly lower than the control plants, due to the low pigment content. No significant differences were found within the control plants. The higher Fv/Fm value is associated with the low chlorophyll-a content *(Figure 3).* It is assumed that the chlorophyll biosynthesis was temporarily inhibited. The carotinoid content was slightly more reduced than that of the chlorophylls. (*Figure 3).*

3.3. Cytology and genetic assay of the semi-shrub mallows interspecific and intergeneric hybrids

3.3.1. The genome size of the mallows interspecific and intergeneric hybrid

We found that *Sida hermaphrodita* has the biggest genome size (7.3 pg) within the examined species and hybrids and the *Alcea biennis* had the smallest size (2.6 pg). The hybrid genome size exceeded the parent species' relative DNA content by 1 pg in the case of *Althea officinalis* and by 1.8 pg in the case of *Alcae biennis*. We could not find significant differences between the *Kitaibelia* hybrids and parents, nor the *Alcea rosea* and the intergeneric hybrid. The differences between the Sida control and polyploid varieties was not significant, Moreover, in some cases higher C-value was measured in the control plants.

3.3.2. Examination of the genetic diversity with genetic markers of the mallows interspecific and intergeneric hybrids.

The leaf morphology and the DNA fragments gained with the UBC-818 ISSR primer is shown in Figure 4. Based on the dendrogram, using the UPMGA method and the calculated genetic similarity, there are marked differences between the *Kitaibelia* interspecific hybrids. Based on the obtained results, the shape of leaves and the petal color is a suitable morphology marker, and it can be used for the marker-assisted breeding of these species. The Aurea varieties did not show a closer genetic connection to each other compared to the other progenies. The reason for this phenomenon is that more mutant parent lines were involved in the establishment of the population.

Figure 2. Genetic similarity matrix of the Kitaibelia interspecific hybrid using UBC primmer and UPMGA cluster analysis method.

	KV	**Kvb-A3**	**Kvb-A6**	**Kvb-R**	**Kvb1-10**	**Kvb2-7**	**Kvb1-25**	**Kvb6-19**
KV	100.00							
Kvb-A3	66.67	100.00						
Kvb-A6	40.00	66.67	100.00					
Kvb-R	75.00	66.67	40.00	100.00				
kvb1-10	75.00	66.67	40.00	100.00	100.00			
kvb2-7	57.14	80.00	50.00	85.71	85.71	100.00		
kvb1-25	66.67	100.00	66.67	66.67	66.67	80.00	100.00	
kvb6-19	40.00	66.67	0.00	40.00	40.00	50.00	66.67	100.00

In the case of the *Alcea x Althea* intergeneric hybrid (“Háros”), significantly less primers are appropriate for molecular marker analysis. The dendrogram gained by the UBC primer shows the genetic relationship between the parent's species and the progeny (*Figure 4*)..

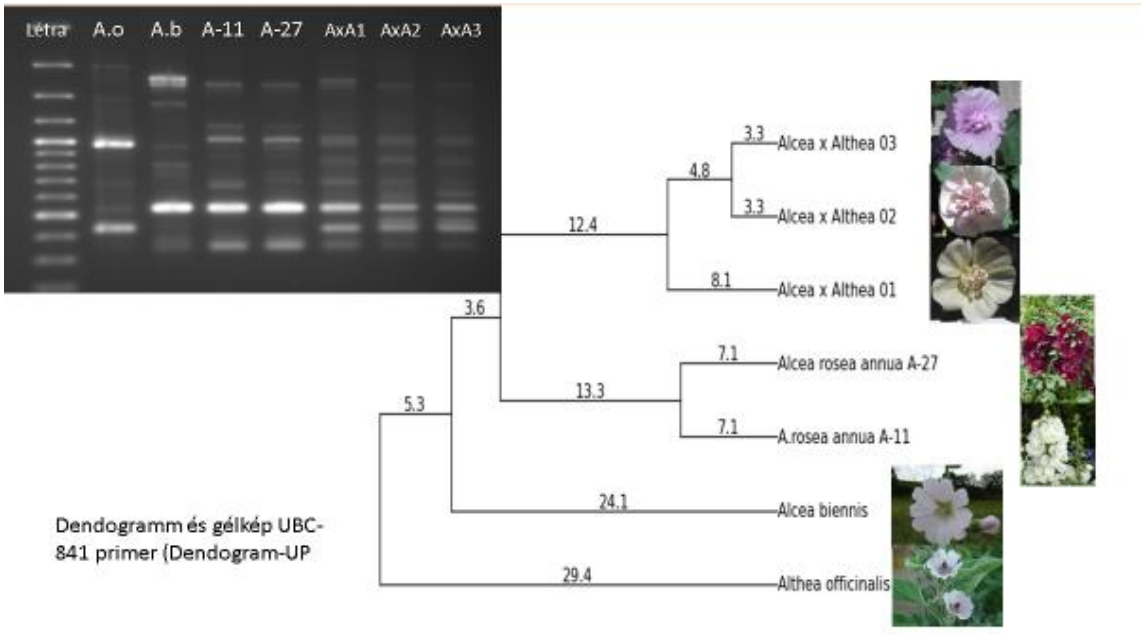

Figure 3. Dendrogram of *Althea and Alcea* intergeneric (UBC-841 SSR, UPMGA)

Legend: Athea xAlcea 01-03 varieties of the "Háros" intergeneric hybrid

3.4. The mallow seeds compound assay

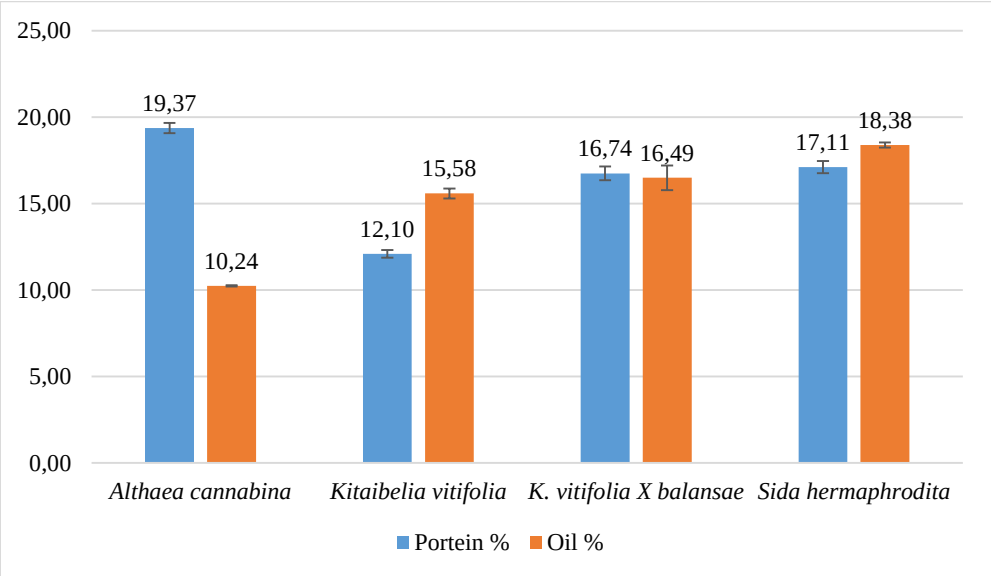

Figure 4. Seeds oil -and protein content of *Kitaibela vitifolia* Willd., a *Kitaibela vitifolia* Willd. x *K. balansae* Boiss. and *Althaea cannabina* and *Sida hermaphrodita*.

Based on the seeds compound assay, it was found that most favorable protein ratio was observed in *A. cannabinas* seeds (19.73 % crude protein) and higher oil content was measured in *Kvb*

seeds (16.5%) within the Althea cannabina K. vitifolia and Kvb seeds. The fatty acid compositions of the *K. vitifolia* seeds were fairly favourable (*Figure 5*) and the ratio if linoleic acid is high enough (omega-6) (68.61%). In addition, the oleic acid content was 14.43%. Minstric acid, arachin acid and gladoil acid contents were below the detection limit. We could not detect significant differences between the *A cannabina and K. vitifolia* from the aspect of fatty acid composition. The composition of seeds of *K. vitifolia x balansae* was also favourable. The most favorable composition of all examined species was that of the sida, but this favorable trait depends on the given season. The fatty acid composition is also beneficial, as it contains a high ratio of linoleic acid and oleic acid (67.45% and 13.14%).

3.5. The result of *in vitro* propagation of Kvb kvb-1-1-1

3.5.1. The in vitro callus development of Kvb hybrid influenced by NAA and KIN

It was found that the intensive callus development is a joint result of both plant growth regulators, NAA and KIN in an optimal ratio. The callus growth ratio was 150% in the media no. 3, while the higher NAA content (0.5 mg l^{-1}) increased the callus size to 230%.
The useable morphogen callus form (embryogenic) was observed in the media no. 3. and no. 4 (*Table 5*), in which the developed calli were not vitrified or did not become white.

Table 3. The morphogenic callus development and the type of callus formed in the different media

	growth regulator content	Expansion %	Callus type*, **
1.	0.2 NAA	52.50±31.12	e++ f++
2.	0.5 NAA	105.63±42.24	e++ f+++ v+
3.	0.2 NAA+0.3KIN	150.00±40.82	e+++ v+
4.	0.5 NAA+0.3KIN	233.33±58.03	e+++ v+
5.	-	146.67±75.87	e+++ f+

*Note: e: morphogene /embryogenic callus, v: vitrified, soft callus, f: white callus, ± SD

**Note: +: the callus type frequency is between 0-25%, ++: the callus type frequency is between 25-50%-, +++: the callus type frequency is more than 50%.

The amount of non-preferable, vitrified, or soft callus formations was lower in these two media (no. 3 and 4), however, the differences and the positive effect was not significant compared to the control (media no. 5, *Figure 9*). Based on the obtained results, the embryogenic callus

induction is possible by using NAA and KIN in combination and in a correct combination in the case of Kvd interspecific hybrids. The optimization of the media and the growth regulator ratio to the genotype should increase the effectiveness of the in vitro embryogenesis. (*Figure 6*).

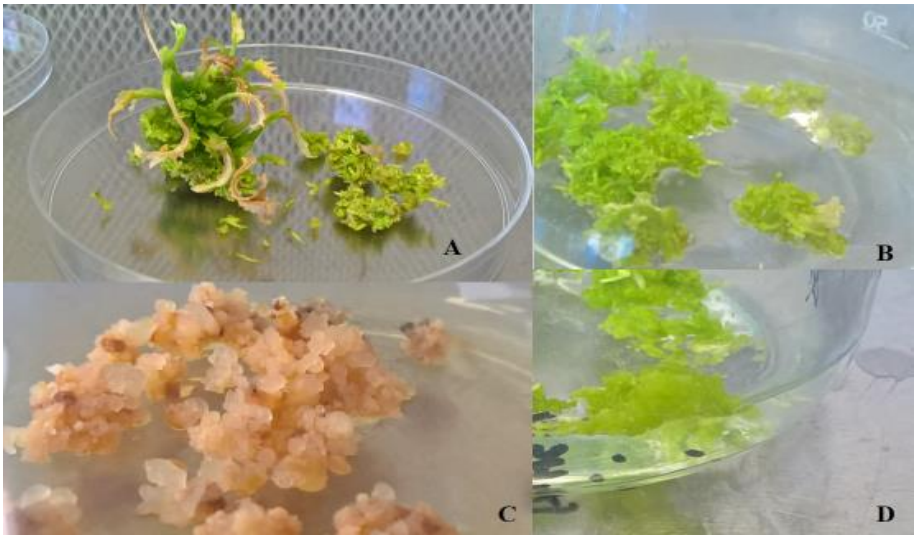

Figure 6. A Kvb in vitro callus formation

Legend: A: mother plant with morphogenic callus, B: Morphogenic callus cluster, C: white callus, D: vitrified and soft callus,

These observations confirmed the preliminary results of Szarvas et al. (2008) research about in vitro propagation of *Althea* and *Alcea* species. In this work, the embryogenic callus initiation was not observed (Szarvas et al., 2008). It should be noted that the exact growth regulator sensitivity is a genetically determined trait and there are many differences between the genotypes, which calls for further investigation.

3.5.2. The in vitro shoot and root formation in different carbohydrate sources and salt concentrations

During the in vitro regeneration experience, the reduced dose of MS salts (50%) and sugar had a positive effect on the root formation on the callus clusters of the Kvb interspecific genotype. The maximum number of shoots emerged from the media supplemented with 1.5% saccharose and 0.1 mg l^{-1} NAA. In addition, the higher number of the plants were regenerated from this medium. Glucose supplementation increased the number of rooted clusters (rooting %), but the number of shoots was lower and the undesirable callus formation was detected. Callus

expansion was a general phenomenon in all medium types. The qualitative callus formation change was caused by half MS salt concentration and the application of glucose. (*Table 6* and *Figure 7*).

Table 6. The effect of reduced carbohydrate and auxin in the in vitro root and shoot development

Media	callus increasing %	Callus type	Number of shoots per explants	Rooting %	complete plants/pd
½ MS 1%+ starch 2%	75±18.02	e++ v+++	5.5±3.9	45±11.1	0.5±0.5
MS 1%+starch 2%	90±14.14	e++ v++	4±1.22	15±20.6	1±0.7
MS 1.5% GLUC+0.1 NAA+SH vit.	82.5±30.31	e+ v+++	4.5± 3.64	45±28.72	0.75 ±0.83
MS 1.5%+0.1NAA+SH vit.	18.75± 7.39	e+++	11±7.11	11.25±5.44	8.75±2.38
½ MS 1.5%+KNO_3+SH vit.	77.5±2.78	e++ v+++	3.25± 2.58	42.5±21.65	0.75 ±0.83

Legend: pd - Petri-dish

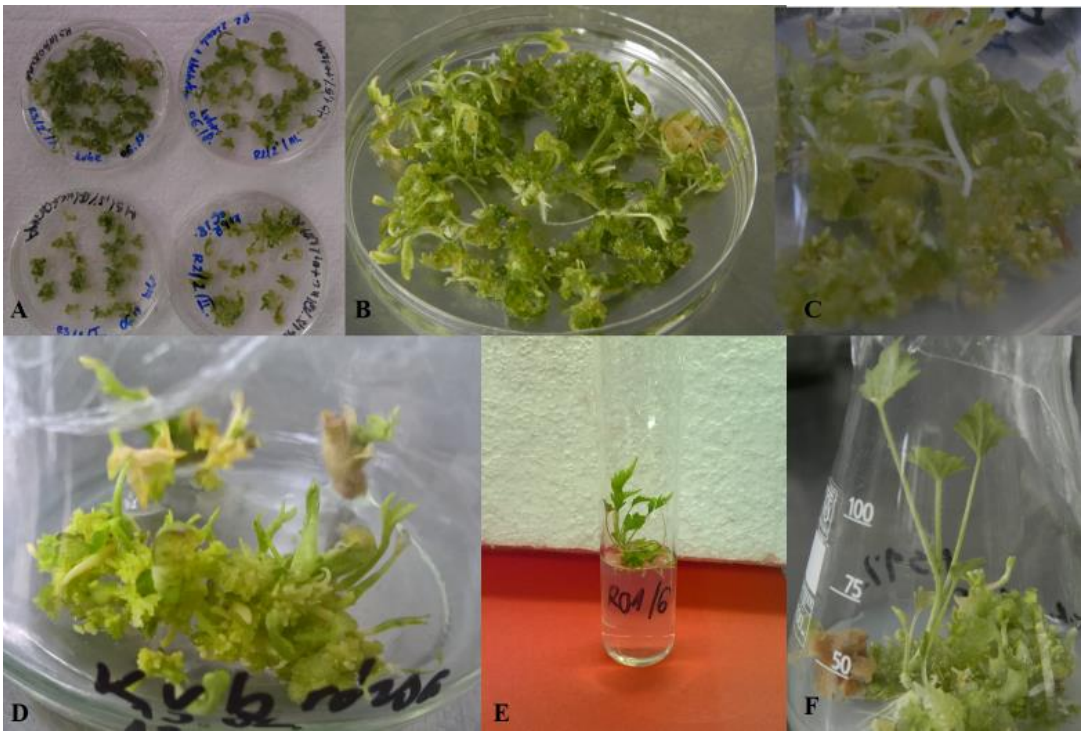

Figure 5. The embryogenic callus regeneration experiences of the Kvb genotype

legend: A: the effect of carbohydrate source on the plant regeneration (upper: media with saccharose, lower with glucose);
B: different maturity embryo clusters developed in the half-strong medium supplemented with 0.1 NAA, 1.5% C: roots and callus developed in a medium supplemented with 1% saccharose and 10 g l^{-1}

starch; D: Regenerated shoots from organogenic callus in hormone-free medium (4 weeks old), E: regenerated plant from 0.1 NAA, 1.5% saccharose medium; F: callus and regenerated plant from a medium supplemented with 1% saccharose and 10 g l^{-1} starch

In this medium, the calli become vitrified, but at the same time, the rooting percentage was higher. The experiences of the reduced sugar effectivity verified the rooting induction effect of the reduced carbohydrate content. We did not gain as successful shoot regeneration using TDZ and BAP in combination as in the case of *H. acetostella* by (Sakhanokho, 2008). Due to the fact that inducted shoot regeneration became more efficient, further investigation of another cytokinins effect, such as TDZ, or metatopolin, needs to be carried out.

3.6. Vegetative propagation of the horticulturally valuable genotypes by means of shoot cuttings

The source of shoot cuttings was the semi-woody region in the middle of the shoots (Figure 8.). The effectiveness of vegetative propagation was low enough (0-47%), which can be increased with changing the date of collecting the cuttings, or by applying any rooting product. We created a breeding stock from a valuable variety.

Figure 6. The breeding stock of Kvb interspecific hybrids (2017, Debrecen).

Legend: The rooted shoot cuttings of the Kvb hybrid; B,C: The shoot cuttings of the selected genotype, D: clones of Kvb1/2/4 genotype (pink)

3.6.1. Results of seed biology assays of Sida hermaphrodita

During the seed priming treatment, the most effective temperature of the hot water treatment was determined firstly. Three temperatures were used (65, 80 and 95ºC), thus, 80 ºC had the most inductive and less reductive impact on the germination percentage. Therefore, seed

separation pretreatment was applied before the hot water treatment was finished in the imbibed seeds. Only one seed germinated within the flow-through fraction (FU), while the infection ratio was very high (90.67%). The germination ratio of the imbibed (S) fraction from the same seed sample was 11.33% and only 32.67% of the seeds were infected. The imbibed seeds had a 6-7% higher germination percentage, and more than 10% less infection ration compared to the control. These imbibed seeds could gain a germination percentage of 80% (79.33%) as a result of the hot water treatment (S-80) and infection was reduced to zero in all replications.

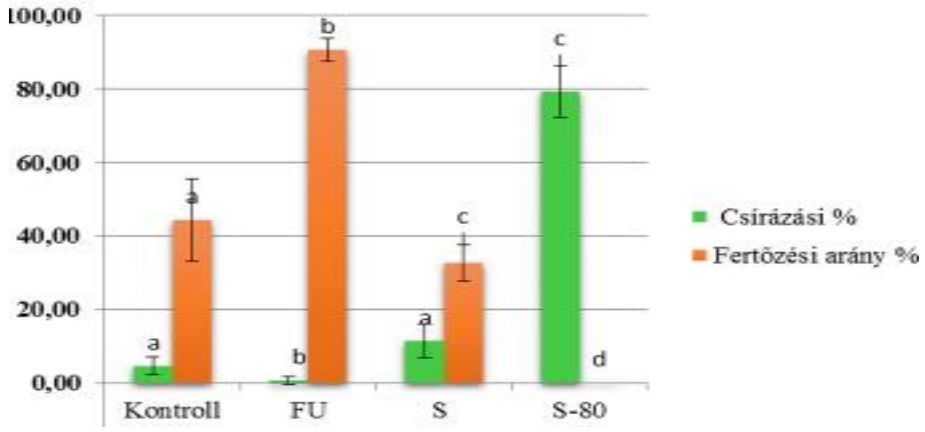

Figure 7. The effect of the two-step seed priming on the germination capacity and the infection ratio.

Legend: Different letters mean that there are significant differences between the measured characteristics at the level of $\alpha = 0.05$ based on the TUKEY test.
K: Control seeds, which were not treated with hot water or separated, just oak in distilled water for 30 min, FU: floated seeds/ the flow-through fraction, S: Imbibed seeds, but not treated with hot water, S-80: Imbibed seeds treated with 80oC distilled water.

3.6.2. The plantlet production of Sida

The Nurse in Tray method

Figure 10 shows the marked differences between the HWT performed at different times after the treatment date. The germination rate of the freshly treated seeds exceeded 60% in the case of a density of 40 seeds per tray. The number 1 treatments were performed 30 days before sowing, when the germination capacity was 24.33-30.8%. The double sprouted plant ratio (where all the double sown seeds germinated) was relatively low (less than 20%). The density of 40 plantlets per tray was the most suitable plant density during plantlet production, in which more densely sown seeds did not inhibit the growth of the plantlets. The seeds treated immediately before sowing showed higher germination capacity and sensitivity to the higher

plant density, due to the more intensive growing potency; i.e., to the competition between the different plantlets (*Figure 10*). No significant differences were found in fresh weight between the plantlets grown in different plant densities.

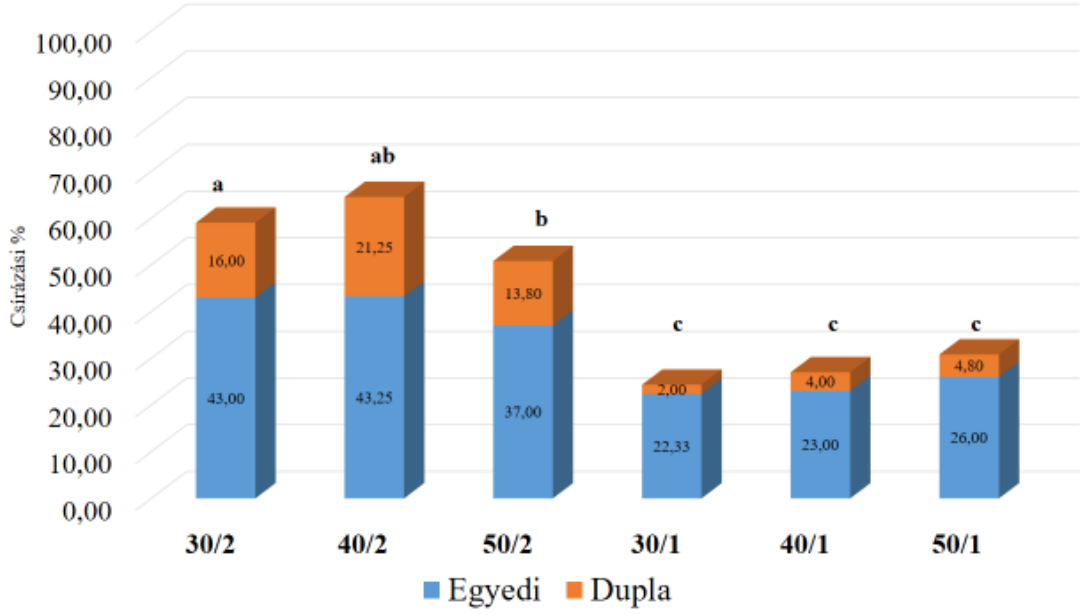

Figure 8. Three plantlet density values in the case of the nurse-in-tray method from treated seeds.

Note: Different letters mean that there are significant differences between the measured characteristics at the level of $\alpha = 0.05$ based on the TUKEY test.

3.7. Biomass production of Sida hermaphrodita

In the second year, 10.2 – 11.8 DM ha^{-1} yield was achieved without fertilization by using extensive technology. This value is within the yield interval referred to in bibliographical sources. The soil exhaustion impact of higher plant density (20 000 plants per hectare) was increasingly prominent in subsequent years of planting. At the same time, the yield obtained with a plant density of 10 000 plants per hectare basically remained unchanged in the three subsequent years. In addition, the wide spacing can make it easier to perform mechanical weed control in the early vegetation period of Virginia fanpetals. However, in the case of extensive technology, organic manure application is recommended in order to provide long-term maintenance. (*Table 7*). In this plot experiment, biomass production decreased signifcantly by the 5th season, which cannot be explained. This reduction was caused by the poliphage fungus disease definitely.

Table 4. The calculated biomass yield (DM) of Sida hermaphrodita, based on the weight of the inner stems (Debrecen)

Season	Density (no./ha)	Inner stems average weight (g)	Average yield (kg/ha)
2011	10 000	300±56	2 996
	13 300	295±53	3 926
	20 000	195±39	3 908
2012	10 000	1121±441	11 209
	13 300	769±225	10 228
	20 000	594±210	11 871
2013	10 000	1126±592	11 265
	13 300	561±201	7 457
	20 000	514±218	10 283
2014	10 000	895±487	8 945
	13 300	427±176	5 674
	20 000	372±251	7 442
2015	10 000	861±614	8 612
	13 300	286±241	3 800
	20 000	211±140	4 218

4. NEW SCIENTIFIC ACHIEVEMENTS

1. The following new valuable interspecific hybrid phenotypes of *Kitaibelia vitifolia x Kitaibelia balansae* population were selected and described: six aurea (Kvb A1-A6), three pink petals, Kvb1/10, Kvb-1/2/4 and Kvb 1/26. The Kvb 1-10 genotype has semi spreading habit, and simple leaves, which have a heart shape, their margins are crenate and their colour was light green The Kvb -1/2/4 genotype is semi spreading, and has simple palmately veined leaves with 3 lobes and acuminate tips, its margins are dentate, their colour is dark green and the base is heart-shaped. The Kvb-1/26 genotype has erected growth habit and the leaves are simple palmately veined with 5 lobes and acuminate tips and the margins are dentate. The leaf base is perpendicular to the petiole and its color is slate green. The Kvb-2/7 genotype has light pink petals and spreading growrh habit. Additionaly, a self cleaning genotype (Kvb-6/19) and a promising high biomass productivity individual (Kvb 2/1/4) were selected.
2. The yellow-leaved phenotypes have reduced PS II efficiency, we measured only 0,1-0,35 Fv/Fm value compared to the controls 0,8 Fv/Fm value, is due to the very low photosynthetic pigment content (chlorofill-a: 1,3-4,7 μg/ cm^2, chlorofill b: 0,9-2,5 μg/ $cm^{2)}$; therefore, the energy demand of initial shoot growing is provided by the nutrient stored during the previous year. Relying upon these findings, we have to consider this phenomenon when the optimal pruning technique is selected using these varieties in public gardens.
3. I determined the relative genome size of the examined species by using the flow-cytometric method. I did not find significant different on the genom size between the *K. vitifolia* (2,26 pg)and the interspecific *Kitaibelia* hybrid (2,33 pg). In case of intergeneric hybrid , the genom size was significant higher (2,22 pg) compared to the two parenthal lines (1C: *A. officinalis*: 1,68 pg, *A. biennis*: 1,32 pg).
4. Within the examined mallow species there is a significant correlation between the imbibition capacity and the germination percentage. Based on these findings, the effective seed treatment technique needs before the introduction of these species to the market. This priming method have to include a selection step as it can increase the germination percentage of *Sida* by more than 60%, and in case of *Althea cannabina* by 8-10 %.

5. Based on the favourable seed composition of the interspecific *Kitaibelia* hybrid (16.7% oil and 16.5% crude protein content) and its advantageous fatty acid ratio (62.4 linoleic acid and 15.2 % oleic acid), it is possible to determine a new breeding direction, due to the heterosis effect in the case of the seed composition, because the interspecific genotype has higher protein and oil content (*K. vitifolia*: Oil:15.5, protein content: 12.1).

5. SCIENTIFIC ACHIEVEMENTS IN PRACTICE

1. *In vitro* cell–plant–cell system development in the case of interspecific hybrids. This method will be essential for the modern molecular genetics-assisted breeding in the future.
2. Based on the flow-cytometric data, different ecotypes and (interspecific or intergeneric) hybrid population comparison, as well as further breeding programme initiation and optimalization (for example polyploidization) will be possible.
3. In case of *Kitaibelia vitifolia x K. balansae* hybrid population nine valuable genotypes (Kvb-2-7,Kvb-1/26, Kvb-1-2-4, Kvb-1-10, Kvb- A1, Kvb-A3, Kvb-A4 Kvb-A5 Kvb-A6) were selected, and propagated by stem cuttings to establish gene bank material for the futher breeding purposes.
4. In the case of Sida, we developed a seed treatment method, which can increase the germination capacity from 5-10% up to 50% under greenhouse circumstances, (56,2% in case of the spring plantlet production, and up to 70% in case of the nurse in tray method) and up to 79% under laboratory conditions. Based on this seed treatment method, the nurse-in-tray method, a cost-effective and programable plantlet production technique was developed.

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Neptun ID: LM58N6
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