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The use of filter paper bridges for initiating *in vitro* culture OF *Viola wittrockiana* F1 Matrix 'Blue Blotch'

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Abstract

Viola x wittrockiana Gams. is known worldwide as an outdoor ornamental plant and it is suitable for gardening. Due to its extremely beautiful flower, increased adaptability and remarkable resistance to different extreme conditions it became intensively cultivated and breeders developed more specific varieties both in vitro and ex vitro. The main purpose of this study was to analyze the in vitro seed germination and the seedling development of the blue variety of Viola x wittrockiana Matrix F1 'Blue Blotch', pursuing two different types of substrates: a classical MS62 solidified culture medium (agar 8 g·l^{-1}) and a liquid culture medium provided with Blidar type filter paper bridges. The analysis of the data results demonstrated that in case of this species the use of liquid culture medium provided with filter paper bridges positively influences the seedling development when considering the following parameters: length of roots, hypocotyl, epicotyl, stem, leaves, number of leaves as well as fresh and dry weights for an observation period of 60 days.

Keywords: *Viola x wittrockiana*, blue variety, filter paper bridges, *in vitro*, plant biotechnology

1. Introduction

Pansy is the common name of *Viola x wittrockiana* Gams., that is an out-door ornamental plant species highly used for a long time in gardening. The species belongs to the Family Violaceae and it was obtained during the XIX century upon the crossbreeding of three wild different species originating from the temperate area (SAEIDI MEHRVARZ & al. [1]). Pansies are perennial herbs and depending on the cultivars may have a height between 10 and 25 cm depending on the moment of observation such as spring, summer or autumn (HOWE & WATERS [2]). The diversity of flowers is impressive with a unique geometry being composed from 5 petals: two upper side, two lateral and one (YOSHIOKA & al. [3]). A series of studies regarding the ex situ proved the influence of temperature on the pansies growth, they show frost resistance and a better growth at low temperatures. Temperature is a very important abiotic factor influencing pansies development, being proved their resistance to frost and even better growth at low temperatures (ADAMS & al. [4], NIU & al., [5]). Pansies are among the most commercialized ornamental plant species due to their diversity of flowers (i.e. the flower shape, dimension and colour) and also due to the fact that express two blooming periods: during spring and autumn, which are advantages compared to other species (LAWSON & al., [6]; KELLEY & al. [7]). The production technologies evolved in time from the traditional to the modern biotechnologies. Plant biotechnologies grounded the further development of new connecting scientific domains such as that of genetic engineering in the scope of producing new varieties and hybrids in a short time expressing resistance to biotic or

abiotic factors, or producing new metabolites or increasing specific metabolites quantities (OKSMAN-CALDENTEY & BARZ [8], PĂUNESCU [9], PERCIULEAC & LEŞANU [10]). Moreover, these biotechnology applications are contributing to the increase of biodiversity in direct connection with Aichi Targets set by the 10th Conference of the Parties of the Convention on Biological Diversity for more than 190 signatory countries to this Convention (ANTOFIE [11]). In the last decade a series of *in vitro* experiments have been design for *Viola x wittrockiana* in order to better understand its multiplication or breeding purposes or both. Thus, in 2007 a protocol for plant regeneration was published, starting from petiole via callus cultivated on a classical MS62 culture medium supplemented with 2,4-D (i.e. 2-4- Dichlorophenoxyacetic acid) and BA (i.e. N6-Benzil adenine). The primary callus was sub-cultivated on new culture medium for plant regeneration (WANG & al., [12]). Using flower anthers it was possible to induce callus production with reduced ploidy cells and it was possible to conclude that there exists a close connection between the level and type of hormones and the expected answers of the inoculum. During the same experiments it has been proven that sucrose concentration is a limiting growing factor (LIU & al. [13]). Furthermore, during the *in vitro* germination of the pansies' pollen, the importance and concentration of sucrose from the culture medium was highlighted in order for it to be maximum (LI & al. [14]). It has been proven that potassium silicate has a positive effect on the *in vitro* cultivation of pansies and begonias, the biomass growth as well as chlorophyll content in the leaves being stimulated (LIM & al. [15]). During the last ten years new techniques have been developed for the initiation and further cultivation of plant tissue cultures for replacing the solid culture medium, which is more expensive compared to the liquid culture medium. Thus, if in the past the MS62 based solid culture medium was mainly used, the use of liquid culture media and filter paper bridges increased during the last years. This technique proved to ensure better conditions for germination and growth, which influences the reduction of the costs and also the environment pollution (BLIDAR [16]). Currently, there are few studies that present comparative results showing the positive or negative influence on using liquid culture media and filter paper bridges compared to solid culture media. Also, there are only a few comparative results highlighting the effectiveness or ineffectiveness of using filter paper bridges for liquid media compared with solidified culture media. One of the first experiments that proved the efficient use of filter paper bridges for *in vitro* cultures was made by Nabors and Mohmand in 1998 for *Triticum aestivum*. They demonstrated that both the total and the embryonic callus showed a significantly higher number of regenerated plants when using liquid culture medium and filter paper bridges compared to the solidified culture medium (MOHMAND & NABORS [17]). Subsequently, ZHONGXU & al. [18] observed a more efficient *in vitro* rooting when using liquid culture medium with filter paper bridges compared with solid culture medium. On contrary, for *Trapa japonica* the shooting was not influenced by the solid culture medium or the liquid culture medium and filter paper bridges (HOQUE & ARIMA [19]). Still, the use of liquid culture media with filter paper bridges induced higher germination efficiency for seeds of *Pogostemon cablin* and *Litchi chinensis* compared to solid culture media (KHAN & AHMAD [20], SWAMY & al. [21]). Along with the above mentioned positive effects, other experiments revealed that using liquid culture media and filter paper bridges positively influence the synthesis of different secondary compounds of metabolism including certain enzymes. For example, for pineapple it was recorded the synthesis of larger quantities of enzymes bromelain (VILANOVA & al. [22]). In 2004 Blidar proposed a new type of filter paper bridges and through a series of comparative experiments conducted on corn and wheat demonstrated the effectiveness of their use in liquid culture media for *in vitro* plants germination and growth compared with agar medium (BLIDAR [23],

BLIDAR & al. [24, 25]). The same type of Blidar bridge has been tested on the ornamental species of *Viola wittrockiana* the red variety, where it was also noted the positive effects compared to solid culture medium (BLIDAR & ILEA [26]). Over time, data proving the inefficiency of the filter paper bridges have been also published. Thus, Bardoza & al. in 2009 observed a lower shooting process for *Ananas comosus* (BARBOZA & al. [27]) or an inefficient growth and development for protocorms-like Bodies (PLBs) belonging to a hybrid of *Cymbidium* (TEIXEIRA da SILVA [28]). Filter paper bridges were also used in experiments where the comparison was with different types of support. Experiments on plantlets' growth of *Cymbidium hibridum,* which were conducted over several years using filter paper bridges Blidar (BFPB), have pursued several issues, such as: the effect of trace element deficiency, the effect of caffeine and fructose (BLIDAR & CACHIȚĂ [29], BLIDAR & al. [30]). BFPB was also used in tracking and regeneration capacity of callus from ornamental strawberry *Fragaria x Potentilla* (ȘUȚAN [31]). Other experiments were carried out on different species such as *Hypericum perforatum* (SAVIO & al. [32]), *Solanum tuberosum* (MIX-WAGNER [33]), *Nicotine tabacum* (MUCCIARELLI & al. [34]), *Medicago sativa* (KALENGAMALIRO & al. [35]) the authors using these supports for accessing several advantages they offer, both in terms of economic and efficiency. The main goal of this study is to present new scientific data regarding the influence of BFPB used for liquid culture media on the in vitro development of the blue variety of *Viola x wittrockiana* F1 Matrix 'Blue Blotch', all scientific data being compared with those obtained from agar culture medium.

2. Materials and Methods

Plant material and conditions for inoculation and growth. The plant material was represented by certified seeds of *Viola x wittrockiana* Matrix F1 blue variey (**VB**) provided by Tg-Mures Agrosem SRL, Romania. The seeds were selected to be intact and uniform in size and colour. *Disinfection.* The selected seeds were first maintained under running tap water for 10 minutes. Seed disinfection was carried out using a sodium hypochlorite solution of 5% commercial bleach (ACE-Auto) for 10 minutes. The disinfected seeds were washed five times in sterile distilled water to completely remove the disinfectant solution. *Inoculation.* Two types of original Murashige-Skoog culture media - MS62 (1962) were used for the inoculation of the seeds: solidified with 7 $g \cdot l^{-1}$ agar - $\mathbf{VB_A}$ and liquid culture media fitted with filter paper bridge Blidar type (BFPB) - $\mathbf{VB_{PB}}$. The solidified culture medium with agar was considered the control. The number of culture dishes was equal for each working version, i.e. 100. *Growing conditions* were also identical for both types of varieties. The containers were maintained in growth room for 60 days under 16-h photoperiod, the white light (with colour temperature of 6,500 K) provided by fluorescent tubes producing an intensity of 20 $\mu M\ m^{-2}s^{-1}$ PAR; the room temperature was maintained at 23 ± 1ºC. *Growth measurements* were made following biometric and gravimetric parameters such as: germination, root length, hypocotyl length, epicotyl length, number of leaves, leaf length, fresh weight and dry weight. We also determined the germination faculty (the number of seeds from a lot able to germinate may be expressed as the percentage of the seeds that germinate in the lot). Measurements and observations were made over 60 days at an interval of 10 days each. *Statistical data analysis*: VB_A was considered the control group and the registered values were taken as references for experimental variant VB_{PB}. All statistical analyses were made using Microsoft Excel. Each experiment was repeated three time.

3. Results

The liquid culture medium and filter paper bridges (test group) compared to the classic solidified culture medium (control group) has intensified the germination and growth of seedlings. This was observed after 60 days of monitoring the experiment on germination of *Viola x wittrockiana* F1 Matrix 'Blue Blotch'. The related results are presented below. *Biometric measurements and morphologic aspects at 10 days*. The first studied parameter was germination. By comparing the two types of groups a higher percentage of germinated seeds in the seeds placed on agar (VB_A) compared with those placed on the filter paper bridges and liquid culture medium (VB_{PB}) was observed, this phenomenon being maintained during the entire experimental period. As regards the *length of the roots,* higher values were noted for all inocula cultivated on filter paper bridges compared to those cultivated on solidified culture medium (control group). On the contrary, the biomass of the emerged seedlings on the solid culture medium was higher compared to that of the seedlings originating from the liquid culture medium and filter paper bridges (Table 1). From morphological point of view there were no microscopically differences between the two analysed groups.

Biometric measurements and morphologic aspects at 20 days. The length of the roots was more accelerated for seedlings germinating on the VB_{PB} (is 13.78% compared to control VB_A). The stem formation was observed as well as of the first true leaves. The *hypocotyl length* was almost double for seedlings germinated on VB_{PB} compared to seedlings germinated on VB_A (i.e. 111.6% higher) (Fig. 1A), but still this difference in absolute values is not significant (Table 1). *Biometric measurements and morphologic aspects at 30 days.* At this experimental time all biometric parameters were higher for seedlings inoculated on the VB_{PB} culture medium compared to the control VB_A. Significant differences were measured for the length of hypocotyl (i.e. 83.57%), epicotyl (i.e. 76.79%) and leaves (i.e. 64.04%). Weight parameters such as: fresh weight and dry weight were also higher for seedlings germinated on VB_{PB} compared to those cultivated on VB_A, the percentage difference being of 35.85% (fresh weight) and 35.71% (dry weight) (Table 1).

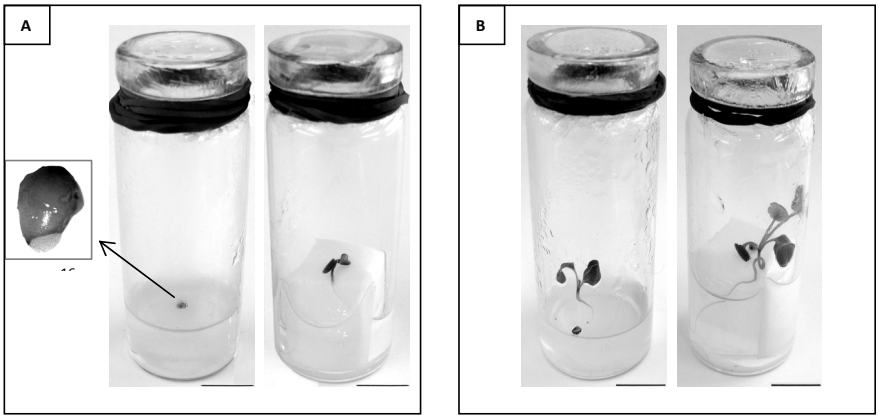

Fig. 1. Comparison of *in vitro* macroscopic aspects at 20 days **(A)** and 40 days **(B)** of *Viola wittrockiana* F1 Matrix 'Blue Blotch'; the seeds was placed either MB-MS culture agarized media (var. $\mathbf{VB_A}$) (control) or liquid media with same mineral and organic composition, in which case sustaining of inocula at the surface of culture media to avoid hypoxia is provided by BFPB, which also fulfilled the role of wick, being in contact with liquid culture medium (var. $\mathbf{VB_{PB}}$)

Biometric measurements and morphologic aspects at 40 days. At this stage significant differences were reported on the extent of rooting and shooting processes among the two groups, the length of roots (i.e. 23.75% higher compared to control), hypocotyl (i.e. 77.44% higher compared to control) and leaves (i.e. 42.93% higher compared to control) for seedlings grown on VB_{PB} being higher compared to those cultivated on VB_A culture medium (Fig. 1B) and the data were statistically supported (Table 1). On the contrary, the fresh weight was lower for seedlings cultivated on VB_{PB} compared to those cultivated on $VB_{A,}$ (i.e. 14.12% higher for control seedlings).

Table 1. Statistical processing of the data measured in the *in vitro* seedlings of *V. wittrockiana* Matrix F1 'Blue Blotch', cultivated on VB_A – solidified MS culture medium (control) and VB_{PB} – liquid MS culture medium with filter paper bridges

		VB_A (control) (solidified media)		**VB_{PB}** (liquid media with filter paper bridge)				
No. of days	**Statistical data / Parameters**	**X ± Sx**	**s^2**	**X ± Sx**	**s^2**	**±d**	**%**	**Signifi-cance**
10	Germination faculty (%)	85.2 ± n/a	n/a	80.4 ± n/a	n/a	-4.8	-5.63	n/a
	Root length (mm)	1.05 ± 0.22	0.05	1.40 ± 0.2	0.04	0.35	0.96	Ns
	Fresh weight (mg)	3 ± n/a	n/a	2.54 ± n/a	n/a	-0.44	-15.33	n/a
	Dry weight (mg)	1.16 ± n/a	n/a	1.09 ± n/a	n/a	-0.07	-6.03	n/a
20	Germination faculty (%)	85.7 ± n/a	n/a	82.6 ± n/a	n/a	-3.1	-3.61	n/a
	Root length (mm)	3.41 ± 0.56	0.32	3.88 ± 0.48	0.23	0.47	13.78	***
	Hypocotyl length (mm)	0.6 ± 0.49	0.24	1.27 ± 0.95	0.91	0.67	111.66	***
	Epicotyl length (mm)	0.19 ± 0.39	0.15	0.19 ± 0.47	0.23	0	0	ns
	Leaf no.	0.09 ± 0.29	0.08	0.05 ± 0.22	0.04	-0.04	-44.44	ns
	Leaf length (mm)	0.21 ± 0.41	0.16	0.05 ± 0.19	0.03	-0.16	-76.19	*
	Fresh weight (mg)	6.94 ± n/a	n/a	4.98 ± n/a	n/a	-1.96	-28.24	n/a
	Dry weight (mg)	1.48 ± n/a	n/a	1.56 ± n/a	n/a	0.08	5.4	n/a
30	Root length (mm)	5.78 ± 0.68	0.47	7.1 ± 0.99	0.98	1.32	22.83	***
	Hypocotyl length (mm)	2.07 ± 0.97	0.95	3.8 ± 0.74	0.56	1.73	83.57	***
	Epicotyl length (mm)	1.81 ± 1.12	1.26	3.2 ± 0.63	0.41	1.39	76.79	***
	Leaf no.	0.52 ± 0.69	0.48	0.7 ± 0.46	0.21	0.18	34.61	ns
	Leaf length (mm)	0.89 ± 0.45	0.21	1.46 ± 0.58	0.33	0.57	64.04	***
	Fresh weight (mg)	8.2 ± n/a	n/a	11.14 ± n/a	n/a	2.94	35.85	n/a
	Dry weight (mg)	1.96 ± n/a	n/a	2.66 ± n/a	n/a	0.7	35.71	n/a
40	Root length (mm)	7.41 ± 1.08	1.17	9.17 ± 1.15	1.33	1.76	23.75	***
	Hypocotyl length (mm)	2.35 ± 1.02	1.05	4.17 ± 0.88	0.78	1.82	77.44	***
	Epicotyl length (mm)	2.7 ± 0.9	0.82	3.28 ± 0.67	0.46	0.58	21.48	**
	Leaf no.	0.9 ± 0.76	0.58	1.25 ± 0.44	0.19	0.35	38.88	*
	Leaf length (mm)	1.77 ± 1.08	1.17	2.53 ± 1.17	1.37	0.76	42.93	**
	Fresh weight (mg)	17.42 ± n/a	n/a	14.96 ± n/a	n/a	-2.46	-14.12	n/a
	Dry weight (mg)	2.25 ± n/a	n/a	5.06 ± n/a	n/a	2.81	124.88	n/a
50	Root length (mm)	8.26 ± 1.31	1.72	11.09 ± 1.71	2.94	2.83	34.26	***
	Hypocotyl length (mm)	2.82 ± 1.18	1.40	4.85 ± 0.71	0.51	2.03	71.98	***
	Epicotyl length (mm)	4.04 ± 1.04	1.08	4.09 ± 1.01	1.03	0.05	1.23	ns
	Leaf no.	2.91 ± 0.79	0.62	2.14 ± 1.55	2.42	-0.77	-26.46	*
	Leaf length (mm)	2.91 ± 1.17	1.38	3.38 ± 0.78	0.61	0.47	16.15	*
	Fresh weight (mg)	19.74 ± n/a	n/a	20.48 ± n/a	n/a	0.74	3.74	n/a
	Dry weight (mg)	4.86 ± n/a	n/a	6.8 ± n/a	n/a	1.94	39.91	n/a
60	Root length (mm)	10.94 ± 1.47	2.16	11.88 ± 1.79	3.22	0.94	8.59	*
	Hypocotyl length (mm)	3.51 ± 0.64	0.41	5.7 ± 0.8	0.64	2.19	62.39	***
	Epicotyl length (mm)	5.05 ± 1.39	1.94	5 ± 1.06	1.13	-0.05	-0.99	ns
	Leaf no.	2.94 ± 0.82	0.68	2.76 ± 1.34	1.81	-0.18	-6.12	ns
	Leaf length (mm)	4.44 ± 1.29	1.67	3.7 ± 0.84	0.72	-0.74	-16.66	**
	Fresh weight (mg)	37.32 ± n/a	n/a	29.46 ± n/a	n/a	-7.86	-21.06	n/a
	Dry weight (mg)	14.6 ± n/a	n/a	10.1 ± n/a	n/a	-4.5	-30.82	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – not applicable.

Biometric *measurements and morphologic aspects at 50 days*. After 50 days the seedling growth follow the same pattern, namely higher values for most seedling parameters when they were cultivated on VB_{PB} compared with the control VB_A. In terms of statistical differences the length of roots and hypocotyl were highly significant (i.e. 34.26% for root length and 71.98% for hypocotyls length – for seedlings cultivated on VB_{PB} - compared to the control – VB_A. Still, the number of leaves was higher for plantlets cultivated on the control culture medium VB_A (i.e. 26.46% more leaves) compared to the tested culture medium VB_{PB} (Table 1). Biometric *measurements and morphologic aspects at 60 days*. Measurements at 60 days from the beginning of the experiment showed a slowdown in growth and evolution of stem development as well as fresh biomass and dry weight accumulation. Moreover, the length of roots and stems for seedlings growing on the VB_{PB} were higher compared to those cultivated on the control VB_A culture medium (Table 1).

4. Discussions

Based on the analysis of these results it can be considered, at least for the macroscopic aspects, that in case of the *Viola x wittrockiana* F1 Matrix 'Blue Blotch', the use of MS62 liquid culture medium with filter paper bridge may have positive effects on seeds' germination and seedlings further development compared to the classical MS62 solidified culture medium. These results further support other previous results published for an earlier study conducted on the red variety of the same species (BLIDAR & ILEA [26]).

An explanation for the improvement of plant growth when the seeds are germinated on the liquid culture medium provided with filter paper bridges is that it could be the constant access to the nutrients around the roots, which is decreasing in the solidified culture media, thus reconfirming previous results. In this second case, nutrients, minerals and organic compounds are trapped in the agar meshes, which once consumed they are not replaced through diffusion and a gradient of concentrations is realized in the solid culture medium with a gap of nutrients around the root system (MAENE & DEBERGH [36], ANTOFIE & BREZEANU [37], BLIDAR [16]). After analyzing the obtained results, we can say that using liquid culture medium with filter paper bridge to support seed had a beneficial effect on seedling growth and development compared with using a classic medium, solidified with agar-agar. The same issues were observed in the case of an earlier study conducted on the red variety of the same species (BLIDAR & ILEA [26]). A first consequence of the easy accessibility to culture medium components is to ensure seeds effective hydration. This was observed by DHABHAI & al. [38], in *Acacia nilotica* and BLIDAR & al. [24, 25] in *Triticum aestivum* L. and *Zea mays* L. ssp. *mays*. If in the this experiment, it was noted that the germination was higher on the solidified medium with 3.61% confirming that it was an effective hydration on solid medium, probably because the size of the used seeds has been reduced (less than 2 mm in diameter), the water demand being therefore small. The effectiveness of using liquid culture medium was analyzed and shown for each stage of seedling growth and development both on stem and roots growth. The same measurements were observed in other experiments for identifying efficiency or inefficiency of using liquid culture media of filter paper bridges compared to solidified culture media. For example, in 2011 an experiment conducted on wheat highlighted the superior efficiency when using liquid medium with filter paper bridges compared to the solidified in the growth and development of roots, leaves and fresh and dry biomass (BLIDAR & al. [24]). Similar results were observed in the study conducted on corn roots where the growth differences for liquid substrate with filter paper bridges compared to the solid, after 21 days was of 290.02% (BLIDAR et al. [25]). On the contrary for strawberry and *Zhumei crabapple* the root growth

period decreased on the liquid culture medium compared to the solid culture medium for a range of 3-10 days, with a growth rate of 4.1% and 20% respectively higher in plants grown on the filter paper bridges compared to those cultivated on classical culture solidified medium (ZHONGXU & al. [18]). Also, a significant increase in roots length was recorded for the cultivation on liquid culture medium provided with filter paper bridges compared to solid culture media, for *Jasminum officinale* L., there being signalled differences in roots emerging on the filter paper bridges and also roots length (BHATTACHARYA & BHATTACHARYYA [39]). Differences between seedling development of *Viola x wittrockiana* Matrix F1 'Blue blotch' grown on liquid culture medium provided with filter paper bridge compared to those cultivated on solidified culture medium were also observed for the followed gravimetric parameters, fresh and dry weights. Higher values were recorded during the first 50 days for seedlings cultivated on liquid culture medium provided with BFPB compared to those emerging and developing on the classical solified medium, the percentage differences being of up to 34.2% in the fresh weight and 60.2% for the dry weight. The same trend was observed in the study conducted on *Helianthus annus* the differences being of 25.5% for fresh weight and of 5.57% for dry weight (BLIDAR [16]).

5. Conclusions

Advantages in using BFPB, for *in vitro* culture can be defined in several ways. In case in which the subcultivation of vitroseedlings is desired a the faster growth and development can shorten the timeframe for sub-cultivation. The roots emerging on the filter paper bridges and, not into the culture medium are suitable for preparing plantlets for the pre-acclimation phase avoiding root damage, if they are developed in the solidified culture medium. All these positive factors can contribute to cost redution in terms of energy and materials further supporting the cost-efficiency of *in vitro* multiplication.

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