CALLUS FORMATION AND PLANT REGENERATION IN SUNFLOWER (*HELIANTHUS* L., ASTERACEAE) IN VITRO TISSUE CULTURE

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ABSTRACT

For the sake of introduction of sunflower to *in vitro* culture the seeds and flowers of different wild species were used: *Helianthus annuus* (three samples), *H. decapetalus*, *H. giganteus*, *H. macrophyllus*, *H. nutalli*, *H. occidentalis* ssp. occidentalis, *H. tuberosus* (four samples), *H. tuberosus* velmoren, and also cultivated variety "Master", lines VIR 114B (fertile) and VIR 114Apet (CMS). Fifteen combinations of plant growth regulators have been used. We were successful in introduction into the culture in vitro and obtain stably growing callus of *H. annuus* wild type (1 sample), *H. giganteus*, *H. occidentalis* ssp. occidentalis, *H. tuberosus* (callus from seeds and from flowers) and sunflower cv. "Master". The optimal medium for callus formation was MS with BAP and NAA (ratio 2:1) or TDZ. We could obtain successful regeneration from callus only for *H. giganteus* on MS media with BAP and NAA (ratio 2:1 or 5:1 in low concentration) when cultivated in the dark.

Key words: Sunflower, *In vitro* culture, Plant regeneration, *Helianthus giganteus*

Abbreviations: BAP - benzylaminopurine, NAA - naphthylacetic acid, TDZ - thidiazuron, AS - adenine sulfate

INTRODUCTION

Different methods for callus induction, adventitious bud formation, shoot multiplication and rooting of *in vitro* formed shoots of sunflower are described (Lupi et al., 1987; Weber et al., 2000; Rath and Pearson, 2004; Ozyigit et al, 2007; Neskorodov, 2011; Sujatha et al, 2012; Khalil et al., 2015 and others). According to the literature it is known that sunflower *in vitro* culture is quite successful, but stable plant regeneration from callus is difficult. The greatest success is achieved using direct regeneration from seed or immature embryos. But the perennial species and interspecific hybrids have very low seed production, or complete sterility (Gavrilova, Anisimova, 2003), so it is difficult to obtain enough amounts of seeds from them. In addition, regeneration by direct somatic embryogenesis (embryoidogenesis) is not suitable for use in genetic transformation protocol (Weber et al., 2000). The second difficulty for finding the optimal protocol of regeneration is a high variability in the response to the conditions of cultivation of species and varieties of sunflower (Ozyigit et al, 2007; Sujatha et al, 2012).

In sunflower investigation the obtaining of a new ways of plants regeneration from callus cultures *in vitro* is of current importance. It is necessary, using different kinds and types of sunflower explants to achieve the production of a stable callus culture, and then to choose the optimal conditions for successful regeneration of plants from callus.

MATERIALS AND METHODS

Helianthus annuus wild type (three different samples), *H. decapetalus*, *H. giganteus*, *H. macrophyllus*, *H. nutalli*, *H. occidentalis* ssp. occidentalis, *H. tuberosus*, *H. tuberosus* velmoren, and also cultivated sunflower *H. annuus* variety "Master", lines VIR 114 B (fertile) and VIR 114 A pet (CMS) were chosen for the study. Seeds were collected from plants grown in the Kuban Experimental Station of VIR.

In most of the experiments we used seedlings obtained from the seeds as primary explants. In addition we used as explants the Jerusalem artichoke (*H. tuberosus*) flowers. Jerusalem artichoke has been chosen as a model, as the most accessible in our region (St. Petersburg and the Leningrad region) perennial sunflower species. Jerusalem artichoke inflorescences at the different flowering stages were collected from the three different samples.

In the first series of experiments, the seeds were superficially sterilized under complete aseptic conditions by soaking in 96% ethanol for 20 min., and then treated over the burner flame for 10-15 seconds. Seeds were germinated for 24 hr on filter paper moistened with distilled water. If at first day of germination the appearance of the hyphae of fungi in the testa was observed, the embryos of these seeds were extracted and subjected to additional treatment of 10% hydrogen peroxide solution in the course of 20 min., then washed twice with sterile distilled water.

Plantlets were transferred to Petri dishes on a ½MS medium (Murashige and Skoog (1962) medium with a half dose of nutrients). Two week later, plantlets were cut into pieces. As primary explants we used all parts of the plantlets: pieces of root, stem, cotyledon, and bud with the leaves. Pieces of plantlets were placed on the MS medium. Three modifications were used, depending on the added growth regulators (MS-1, MS-2, MS-3 - see table). The plates were cultivated at t + 22°C in the dark or light. Then callus was transplanted to five variants of culture medium (MS-4, MS-5, MS-6, MS-7, MS-8). The plates were cultivated at t + 26°C in the dark or under 16 hour photoperiod.

Table. The composition of growth factors added to the culture medium

Growth factors (mg/l)	MS-0	MS-1	MS-2	MS-3	MS-4	MS-5	MS-6	MS-7	MS-8	MS-9	MS-10	MS-11	MS-12	MS-13	MS-14	MS-15
BAP		2		0,1		2	4	4	4	0,2	0,5	1	1	0,5	1	
TDZ			2		2	1	1	1	1							
NAA		1		0,1		0,5	1	1,5	2	0,1	0,1	0,5	0,02		1	0,5
AS				40	40											

In the second series of experiments the tubular flowers were used from the inflorescence of Jerusalem artichoke as explants. The inflorescences were sterilized during 20 min. in 10% solution of hydrogen peroxide, and then transferred to distilled water, disassembled into individual flowers, which were placed for 15 min. in peroxide solution, then washed twice with sterile distilled water. We planted them on five variants of MS medium (MS-4, MS-5, MS-6, MS-7, MS-8).

For plant regeneration, the calluses derived both from seedlings and from flowers were transplanted to a new variant of culture medium (MS-9, MS-10, MS-11 and MS-12). The plates were cultivated at t + 26°C in the dark condition or under 16 hour photoperiod.

Callus with regeneration zones was transferred into a new version of the medium (MS-13, MS-14 and MS-15) to support the growth and rooting. Then, plants both having roots and without them were transplanted into larger flasks on medium without hormones (MS-0).

Subsequently, the plants were transferred from the sterile culture into non-sterile conditions. They were transplanted to pots filled with sand and placed in a climatic chamber at t= + 26°C under 16 hour photoperiod.

RESULTS AND DISCUSSIONS

We had introduced into the culture *in vitro* and obtained stably growing calluses of *H. annuus* wild type (1 sample), *H. giganteus*, *H. occidentalis* ssp. occidentalis, *H. tuberosus* (callus from seeds and from flowers) and *H. annuus* cv. "Master" (Fig. 1a,b,c: 2a,b).

We are faced with the fact that it is difficult to obtain sterile seedlings using freshly collected seeds. These seeds germinated worse and were poorly sterilized. Frequently the re-treatment of emerged embryos was necessary and so we released them from the seed coat, which was struck by mildew, and sterilized again.

To obtain a stable callus cultures from the seeds the following protocol was the most productive: 1) sterilization of seeds, 2) germination on filter paper moistened with distilled water, 3) re-sterilization and transfer of seedlings on a medium ½MS, 4) using all parts of the seedling (root, leaf, stem, cotyledon) as explants, 5) culturing in the dark condition or under 16 hour photoperiod (the second is preferable) and 6) regular transplanting every 6-8 weeks (Fig. 1c,d).

We observed intensive formation of a new callus on MS-1, MS-2, MS-3 or MS-11 media with equal success. So, callus was formed well on media containing NAA and BAR in the ratio 2:1 or in equal low concentration (0,1 mg/l) with AS, as well as on media with TDZ.

The appearance of callus formation centers was noted 7-10 days after transfering. Intensive callus formation continues from 2 to 2,5 months.

Callus formation was observed in different parts of seedling (from pieces, cotyledon, stem, leaves, roots, buds) (Fig. 1d,e).Cytology showed that in callus histogenesis, the formation of centers of meristematic activity and elements of the vascular system occur (Fig. 2h).

In the case of using the tubular flowers as a primary explants the optimal procedure for obtaining a callus is shorter: 1) surface sterilization of inflorescences, then isolation and sterilization of flowers, 2) culturing either in the darkness or under light and 3) regular transfers every 6-8 weeks.

We used for callus induction the five variants of culture medium (MS-4, MS-5, MS-6, MS-7, MS-8). Of these, only a MS-4 medium was bad for callus formation.

Within 7-14 days after the transfer of flowers on a medium, we observed changes similar to flowering in the wild - the disclosure of the tops in anthers (but pollen did not fall out, in contrast to the natural condition). 4-6 weeks later, the beginning of callus formation is noted which occurs intensively within 2,5-3 months, then the callus growth stops (Fig. 2c,d,e).

Callus initiation was observed at the bottom of the disc nectar, and along the upper edge of the tube sepals from anther and stigma tissues. Cytological study showed that in these parts the sections of tissue with small meristem-like cells with dense cytoplasm and nucleus are still persist, while the surrounding tissue consists of large vacuolated cells with badly viewed nucleus (Fig. 2f,g).

We can get the successful regeneration from callus only for *H. giganteus* on MS media with BAP and NAA (ratio 2:1 or 5:1 in low concentration) when cultivated in the darkness. More than one year passed since the seeds have been planted in a Petri dishes and callus was transplanted several times into new versions of the nutrient medium. In the medium MS-9 there were observed gemmo- and rhyzogenesis (shoot and root formation) and rhyzogenesis (root formation), on media MS-10 and MS-11 only gemmogenesis (shoot formation) (Fig. 1f,g,h).

In general, media supplemented with BAP and NAA in ratio 2:1 had a higher callus formation (MS-1, MS-11) as well as media with TDZ (MS-2) and with AS plus low concentration of BAP and NAA (MS-3).

Interestingly, the same range of concentration of 1,0mg/l BAP combined with 0,5mg/l NAA was reported by other researchers to give the highest shoot regeneration (Knittel et al., 1991; Baker et al., 1999; Rath and Pearson, 2004; Ozyigit et al., 2007; Khalil et al., 2015). Some of them noted the

importance of auxins/cytokinin balance for sunflower regeneration, but in our experiments for most samples of sunflower on media with BAP/NAA in such concentration we could get only callusogenesis.

In general it is worth paying attention to presence in cultural medium BAP and NAA in 2:1 ratio, as the most interesting in respect of stimulation of processes of a morphogenesis (callusogenesis, histogenesis, gemmogenesis, rhyzogenesis and a gemmorhyzogenesis) in sunflower culture *in vitro*.

Thus, in literature the different successful protocols which marked for this or that genotype of sunflower are noted, but there is no uniform scheme guaranteeing the success of micropropagation of any investigated sunflower varieties. The response to influence of growth factors depends strongly on the genotype chosen for experiment and is not predictable in advance. Also, the result depends on the choice of the explants and culture conditions (see review in Khalil et al., 2015). Here you can pay attention to more studied culture – corn for which the genes responsible for regeneration *in vitro* are already allocated (Tomes and Smith, 1985; Armstrong et al., 1992; Checheneva and Trukhanov, 1994 and others). Probably, sunflower has similar genes responsible for ability of cells to differentiate on the way of gemmo- or embryoidogenesis *in vitro*. There are works where the positive influence of alleles of several genes to *in vitro* morphogenesis of sunflower is found (Kostina et al., 2013, 2015).

Figure 1. Callus formation and regeneration of plant from seeds. ***a*** **–** *H. giganteus* plants and flower, ***b*** **–** *H. annuus* cv. Master, ***c*** **–** seedling in vitro, ***d*** **–** different parts of seedling in vitro, ***e*** **–** callus formation and rhyzogenesis in primary culture after 4 months, ***f*** **–** gemmogenesis after transferring on new medium, ***g*** **–** callus with shoots and roots after one year, ***h*** **–** new plant of *H. giganteus* in the pot. Scale bar **–** 5 mm

Figure 2. Callus formation from tubular flowers. ***a, b*** – *H. tuberosus* plants and flower, ***c*** – tubular flowers placed on Petri dishes in vitro, ***d*** – three different stage of callusogenesis, ***e*** – callus on tubular flowers after 2 months, ***f*** – longitudinal section of tubular flower (*a* – anther with pollen grain, *o* – ovary, *s* - stigma), ***g*** – zone of callus formation on anther stalk, ***h*** – formation of elements of vascular system in callus.
Scale bar: c, d, e – 5 mm, f – 100 mμ, g, h – 50 mμ.

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