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ARTICLE

Callus Cultures Of Beans Infected With Virus As A Model For Testing Antiviral Compaunds

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ABSTRACT

In the work, bean callus raised from a leaves of *Bean common mosaic virus* infected bean plant was obtained and adapted for the testing of antiviral activity of liposomal glycan-glycolipid complexes. *Ganoderma adspersum* glucans and *Pseudomonas spec*. rhamnolipids were constituents of liposomal compaunds. It has been shown that under the long-term cultivation (up to 3 months) in the presence of a liposomal preparation containing (10-100 mg/l), the virus is eliminated from the tissue. This is evidenced by the absence of 391 bp sequence amplification product established by RT-PCR in the callus tissue, cultured on a medium containing the liposomal complex. The proposed model system is analogous to plant tumors and has obvious advantages over similar systems *in vivo*, since the callus growth is controlled and independent of environmental factors.

1. Introduction

Somatic plant cells, grown under normal conditions, integrate into specialized tissues and organs performing different physiological functions. However, under the influence of certain environmental and anthropogenic factors (injuring, insect and infectious invasions), plants are capable of forming tumors in the form of growths, crowns galls, plant leaf deformations, etc. One of the types of cell neoplasms is tumors induced in plants by the *Agrobacterium tumefaciens* Ti-plasmid [1]. Tumors caused by this pathogen are common in berries, fruit trees, grapes and some field crops and can be modeled *in vitro* in the parenchymal tissues of some vegetable plants and potatoes [2]. In this work, we showed high antitumor and antiviral activity of glycan-containing preparations. Since abnormal plant growth induced by external agents (viruses, bacteria, fungi, insects) as well as by physical and chemical means are actively discussed in literature, the testing of antiviral activity of such substances in callus culture is of particular interest. Thus our earlier studies have shown the possibility of using callus culture (as a species of such tumors) as a model for the selection and study of the mechanism of action of antitumor preparations [2].

This paper deals with tumors formed by meristematic cells that are generated after mechanical wounding of

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cambium, and can also be cultured on artificial/synthetic medium with subsequent regeneration of new plants under the plant growth regulators (auxins and kinetins) influence [1]. In our laboratory, it has repeatedly been shown the possibility of eliminating the virus in case of cultivating a virus-infected callus and obtaining healthy and virus-resistant plants-regenerants [3]. The use of such a method of plant recovery is especially relevant for plants with seed transmission of plant viruses as it was shown for beans infected by bean common mosaic virus (BCMV) and bean yellow mosaic virus (BYMV) [4,5].

The purpose of our research was to: develop a callus cell culture (CCC) for BYMV or BCMV infected bean and try to recover it from viral infection using liposomal glycan preparations (LPs) developed in our laboratory [6].

2. Materials and Methods

2.1 Callus Cell Culture

4-8 week-old seedlings of bean plants *P. vulgaris* cv. Red Riding Hood were grown in spring and summer period in a greenhouse with a temperature cycling between 18 and 24°C. The plants were inoculated by rubbing of extracted sap (in cold 0.1 M phosphate buffer, pH 7.2 (1:10 w/vol) onto leaves pre-dusted with carborundum. Bean plants infected with BCMV or BYMV and showing suspected virus symptoms patterns of mosaic, yellowing, mottling or chlorosis were used for callus formation (Figure 1).

Bean leaf and stem internodes used as the starting material were excised from the mother plant and washed with running tap water. Surface sterilization was done within a laminar air flow cabinet by dipping the runner tips in 70% ethanol (2 min) and 3.0 % sodium hypochlorite (NaOCl) for 3-5 min. Afterwards, explants were rinsed several times with sterile distilled water. The explants (leaves and nodal segments sliced into 0.5-1.0 cm^2 pieces) were cultured on Gamborga (GB) nutrient mediums containing kinetin and 2.4-D at a concentration of 0.1 and 1.0 g/l, respectively. The pH was adjusted to 5.5 before adding agar and autoclaving (121 °C, 0,5 MPa, 60 min). The culture vessels containing explants were incubated in a growth chamber under a 16/8 h light/dark cycle at 25 ± 2 °C. During 1-3 months the observations were made on explant development and all calli were screened for the presence of virus by RT-PCR. The culture passage was performed once a month.

2.2 Antiviral Praparations

Liposomal-based glycan preparations (LPs) were obtained according to previously described methodology [6]. In this work, we used two liposomal glycan-glycolipid complexes (GGK-3 and GGK-4), formed on the basis of the water-soluble glucan *Ganoderma adspersum* (GGK-3), and mix of three glycans (*Candida maltose* mannan, *Ganoderma adspersum* glucan, *Tremela mesenterica* glucuronoxylomannan) (GGK-4). Methods of obtaining and properties of polysaccharides were described earlier [7]. Rh-1 and Rh-2 rhamnolipids extracted from *Pseudomonas spec*. PS-17 culture fluid were used for LPs obtaining. Different concentrations of GGK-3 and GGK-4 (10-500 mg/l) in the form of an emulsion were added to the cultural mediums. Mediums without test substances served control in antiviral activity experiments [6].

2.3 Virus Detection and Identification

Bean leaf samples and callus tissues were assayed by the Reverse Transcription and Polymerase Chain Reaction (RT-PCR). Presence of virus RNA in the calli was assayed every transfer. Total RNA was isolated using AmpliSens Ribo-Sorb DNA/RNA extraction kit. PCR test kit AmpliSens Reverta-L-100 was used to generate cDNA according to the manufacturer's instructions. The reaction mixture for the PCR (of 20 µl) contained: 1 × PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 - 50 ng of cDNA, 0,5U Taq polymerase. Primers were used at final concentration of 5 pmol. The amplification was performed in DNA Thermocycler "Tertsyk" TP4-PCR-01.

The primer pairs chosen for BCMV detection amplified DNA fragments comprising 391 bp of 5'- coat protein region [8]. BYMV1f and BYMV2r primer pairs to the site in the coat protein sequence were used in the study for the BYMV detections [9]. Synthesis of primers was made by Biolabtech (Kyiv, Ukraine).

For the specific primers, amplification was used for 35 cycles: denaturation at 94°C for 5 min, 35 cycles of amplification (94°C for 30 s, 60°C for 30 s, and 72°C for 30 s), and a final extension at 72°C for 7 min. The PCR fragments were verified in a 1.5% (w/vol) non-denaturing agarose gel after ethidium bromide (0.5 mg/ml) staining. The gel was run at 120 volts and maximum current for 45 min before being viewed under UV light and photographed.

3. Results

In Ukraine fairly high incidence of three virus diseases occurs on bean crop. Soybean mosaic virus (SMV), bean common mosaic and bean yellow mosaic viruses have been found to reduce yield and adversely affect seed quality [4,5]. BYMV is the most destructive disease of bean. The virus is widespread and causes economic damage in susceptible bean cultivars that react with apical bud necrosis, leading to plant death [10]. In addition, the virus is spread by a number

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of aphid species non-persistently as well as being seed and mechanically transmitted. In view of these facts, before studying antiviral activity of liposomal preparation in plant tissue culture, we aimed to develop a suitable model system "virus – CCC", firstly, for BYMV.

To obtain the virus callus BYMV-infected bean plants exhibited mild to severe yellow mosaic symptoms on leaves were used (Figure 1).

Figure 1. Callus derived plants of *Phaseolus vulgaris*, infected with BYMV

Leaf blades were cultured on GB basal medium to study their callus induction ability. The explants formed white friable callus after 2 weeks of culture. Results obtained are illustrated in Figure 2. The results reveal that leaf segments yielded a mass of compact white soft callus with a smooth, wet-looking surface (Figure 2A) that turned pink-yellow over time. In 20-23 days of incubation callus induction rates decreased significantly, callus started to be compact and granular (Figure 2B). The explants turned to yellow in color following two weeks of incubation. With time calli growth was stopped then tissues were dried out, necrotized and wrinkled showing the sign of dying off (Figure 2C). The features of proliferation of callus originally raised from the BYMV-infected bean leaves, as well as the low growth rate of callus tissue, are probably due to the high pathogenicity of the virus in bean plants, which significantly restrained the growth of callus.

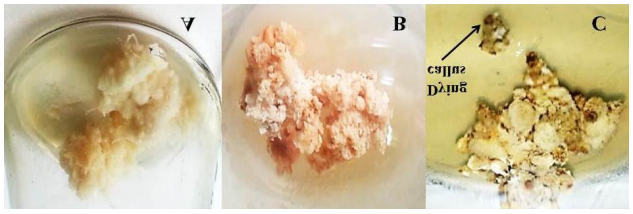

Figure 2. Callus initiation from leaf segments: young calluses after 3 weeks of culture (A); an aging 6 weeks culture (B); later stage of callus incubation (C)

The results obtained indicate that this model system is not suitable for further research on the study of antiviral substances. Therefore, in further work, we focused on the BCMV, as a possible component of the experimental system. According to our observations [4], BCMV is less pathogenic then BYMV and is transmitted at a high frequency through seeds. This circumstance made it possible to obtain virus-infected callus from the 2 sources of explants – leaf and seed germs. Therefore, there was a real opportunity to recovery bean seedling grown from infected seeds. As in the previous case, for callus formation it was selected the bean plants with characteristic symptoms of the BCMV infection – light and dark green systemic mosaic, rugosity, upward and downward leaf curl, stunted growth, leaf roll and malformation of leaves. As a control we used callus raised from a healthy (uninfected) bean plants.

Presence of BCMV in symptomatic plants (Figure 3) was confirmed with polymerase chain reaction (RT-PCR). The amplification of the CP gene from tissues of infected plants with BCMV primers generated a single DNA fragment of the expected size (~391 bp). Amplification did not occur in the control samples (Figure 4).

Figure 3. Callus derived plants of *Phaseolus vulgaris*, infected with BCMV

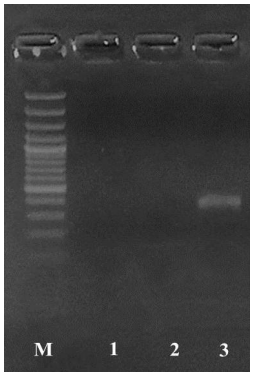

Figure 4. Agarose gel electrophoresis of RT-PCR amplification products

Note: lane 1 (M), 100-bp marker; lane 2, negative control; lane-3, ex-

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tracts from healthy bean; lane-4, extracts from symptomatic bean; expected BCMV band = 391bp

Callus, raised from BCMV-infected bean leaf, seed germ and stem internodes was cultured in a thermostat at 24-25° C for two weeks, and therefore in a luminostat under a photon flux density of 50 µmol/m^2/s, emitted from 'Fluora' fluorescent lamps up to 2.5-3 months. Callus initiation was observed in a week after the explants were placed on media. Generally, the callus tissues grew quite intensively and did not differ in all variants of the experiments (leaf or stem internodes, virus-infected and virus-free) at the beginning. The effective formation of callus was observed in all cases. With time callus proliferated into pale yellow and became compact (Figure 5).

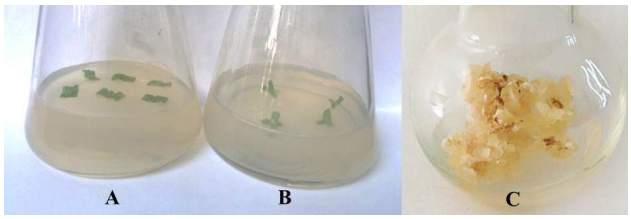

Figure 5. Callus induction on leaf explants (A) and stem internodes (B). Yellow 4-week-old calli initiated from leaf explants. All calluses were raised from BCMV-infected bean

During the experiment, it was found that formation of the derived from different sections of one leaf occurs unevenly: no notable callus formed in a number of explants obtained from infected plant. Eventually explants were died without forming primary callus. In other cases the calluses having translucent pale yellow or white peripheral tissue with greenish inner tissue grew and were not watery in consistency, somewhat fragile, but with a dense inner part. Translucent tissue gradually became dark yellow or orange with brown and relatively compact inner core in later stage. Subsequently, the cell growth stopped, but degenerative processes were not observed.

Antiviral activity of liposomal glycan preparations was measured by inhibitory effects of BCMV replication in cell culture and has been used to evaluate their efficiency *in vitro*. For this purpose we first examined toxicity of LPs to callus cells. According to our previous experiment GGK-3 and GGK-4 application at concentrations in the nutrient medium (500 mg/l) led to severe toxicity development. The date obtained indicates the negative impacts of even at relatively low concentrations of GGK-4 for callus cells and their viability (data not shown). Therefore, in further experiments only GGK-3 was tested at low concentration (100 and 10 mg/l).

Our findings demonstrate that exposure to 10 mg/l of GGK-3 did not decrease the relative growth rate of callus and as a result did not show a clearly pronounced inhibitory activity on callus tissue formation *in vitro* compared with the control (Figure 6). As can be seen from Figure6, morphogenic responses leaf explants exposed to lower concentrations of GGK-3 (Figure 5) and normal (Figure 6) conditions did not differ. The effective callus formation with large cell colonies was observed on leaf explants in all variants of the experiments. The calli grown in both control and experimental groups were composed of translucent and soft inner tissue with white or cream-colored and compact peripheral tissue. This compound at a concentration of 100 mg/l was also slightly toxic. It should also be noted that the most suitable for obtaining the CCC is the explants obtained from the internode and the leaf sections along the central vein.

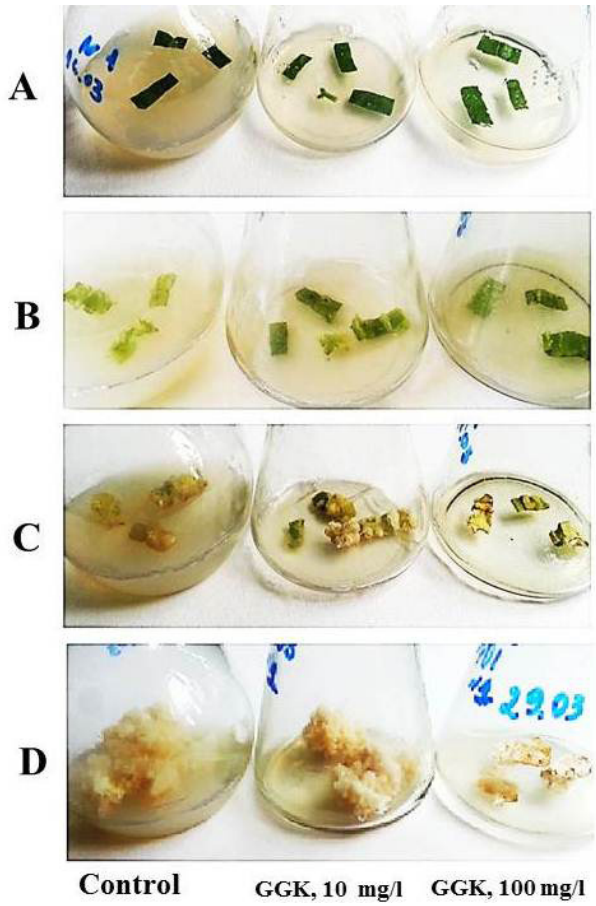

Figure 6. Proliferation of callus obtained from BCMV-infected leaf after 1 day of culture (A), 6 days of culture (B), 13 days of culture (C), 33 days of culture (D)

Thus, in callus tissue growing on the Hamburg B-5 medium with the liposomal glycan-glycolipid complex at a concentration of 10-100 mg/l, probably, a gradual (during 2-3 month) elimination of the virus occurs (Figure 7).

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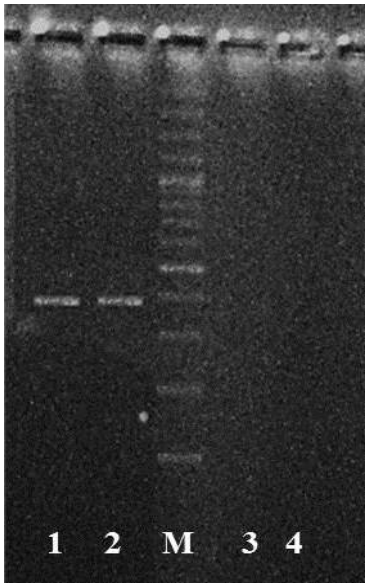

Figure 7. Agarose gel electrophoresis of RT-PCR amplification products

Note: lane 1, starting leaf; lane 2, callus on medium without GGK; lane 3 (M), 100-bp marker; negative control; lane 4, callus on medium with GGK 0,1 mg/l; lane 5, callus on medium with GGK 0,01 mg/l; expected BCMV band = 391bp

These data obtained can be useful for efficient eradication of various viruses from almost all of the most economically important crops and cultivation of virus-free plants as one of the important approaches in novel viral disease control strategy.

4. Discussion

Viruses cause many important plant diseases and are responsible for significant losses in crop. The most unprotected are plants capable of transmitting viruses from generation to generation by seeds or through vegetative propagules. These viruses include Bean yellow mosaic virus and *Bean common mosaic virus* that affect leguminous plants beans [4,5]. Many important horticultural and agronomically important crops are routinely freed of viral contamination using tissue culture procedure combined with chemo- or thermotherapy.

The tissue culture technology is being improved all the time for the mass propagation of plant, but *Fabaceae* plant are lagging behind due to their recalcitrant nature to *in vitro* techniques [11]. By this time the using of environmentally friendly preparations in the tissue culture technology is unknown.

In this work, to obtain the virus-free plant we attempted to combine callus cell culture technique with the using liposomal preparations. For testing antiviral activity of LPs it was selected nutrient media that is most appropriate for bean somatic tissue cultivation. Also it was shown the possibility of virus elimination from callus in the presence of liposomal forms of glycans.

Earlier Shcherbatenko and Oleshchenko reported [3] that under the long-term cultivation, tospoviruses can be eliminated from infected callus. As shown previously, glycans obtained from yeasts and higher *Basidiomycetes* mushrooms, can inhibit viral infections and activate nonspecifc defense mechanisms in host plants as well as suppress tumor growth induced by *A. tumefaciens* [2]. In this study liposomal forms of glycans were used at the first time for *in vitro* methods for plant virus eradication. Their high efficacym as a means of controlling plant viral diseases was confirmed on the callus cell culture [6]. In general, our and literature data demonstrate, on the one hand, the nonspecificity of the action of glycans and glycan-containing complexes against pathogens, and, on the other, some analogy of transformed pathogens with undifferentiated (meristematic) plant cells. This makes it possible to use for practical purposes a system with such features of plant cell cultures - as universal models for screening or studying antiviral and antitumor properties of different compounds. The authors hope that further work in this direction will allow to develop a new technology for cultivation of virus-free plants and new effective means against viral, fungal and bacterial diseases.

Conflicts of Interest

The authors indicate no potential conflict of interests regarding the publication of this paper.

Acknowledgments

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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