

## INTERFERON APPLICATION CAUSES CANOLA SEEDLING BIOMASS INCREASE

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**ABSTRACT**

In order to study canola (*Brassica napus* L.) *in vitro* seedling growth after seed imbibition by human recombinant interferon alpha 2b (INF) solution, the measurements of fresh weight (FW), total soluble protein (TSP) content, and superoxide dismutase (SOD) activity were conducted in 7-day-old plantlets. INF applications did not improve canola seed germination. However, FW of seedlings increased by 100% ( $10^2$  IU/ml INF), 140% ( $10^3$  IU/ml INF), and 70% ( $10^4$  IU/ml INF) in comparison with controls (treatments by pure water, INF after boiling, or INF with diethylthiocarbamate). Increment of FW was detected in hypocotyls and roots. TSP content was not affected in cotyledons and decreased in hypocotyls. SOD activity increased up to 2.18-fold in cotyledons and 1.47-fold in hypocotyls. Human recombinant INF alpha 2b application caused an increase in canola seedling biomass (up to 2.4-fold) during *in vitro* growth and we suppose that it was regulated via increased SOD activity.



**Keywords:** *Brassica napus*, fresh weight, interferon, superoxide dismutase activity, total soluble protein

### INTRODUCTION

Interferons (INFs) are proteins induced in vertebrates by viruses, double-stranded RNA, and some other agents (Staeheli, 1990). It was shown that unfractionated human leukocyte INF, as well as highly purified subspecies of this INF (alpha and beta), and a purified recombinant human leukocyte INF produced in bacteria were active in suppressing multiplication of tobacco mosaic virus (TMV) in tobacco (*Nicotiana tabacum* L.) leaf discs (Orchansky *et al.*, 1982). INF-gamma application increased cytokinin activity and induced synthesis of pathogenesis-related and heat shock proteins in tobacco and wheat (*Triticum aestivum* L.) tissues (Kulaeva *et al.*, 1992). Human INFs (alpha, beta, and gamma) induced antiviral activities in datura (*Datura stramonium* L.) infected by TMV and globe amaranth (*Gomphrena globosa* L.) inoculated by potato virus X (Vicente *et al.*, 1987). Some studies were also conducted for genes encoding interferons (Smirnov *et al.*, 1991, Truve *et al.*, 1993) or interferon-induced enzymes such as the human double stranded RNA-dependent protein kinase (PKR) (Lim *et al.*, 2002) the expression of which improved virus tolerance in plants. Numerous researches were focused on expression of recombinant INFs in plants (Arlen *et al.*, 2007, Sindarovska *et al.*, 2010, Luchakivskaya *et al.*, 2011, Hosseini *et al.*, 2012), however its effect at the whole plant level under non-stressed conditions is not yet sufficiently explored.

In animal cells, exogenous INF-alpha application is accompanied by superoxide radical formation and growing superoxide dismutase (SOD) activity. Thus, preincubation of intact human neutrophils with INF-alpha and subsequent stimulation with calcium ionophore A23187 significantly enhanced superoxide radical generation that reduced nitroblue tetrazolium into blue formazan (Al-Shabanah *et al.*, 1999). Mouse (L, L929, L1210 S6, and L1210 R3) and a human (WISH) cell lines pretreated with homologous INF and different concentrations of diethylthiocarbamate (DDC) for various periods of time were also tested for their ability to support virus multiplication. Treatment of cells with DDC resulted in a dose- and time-dependent inhibition of SOD activity and, simultaneously, in the reduction of antiviral protection by exogenous INF (Pottathil *et al.*, 1981). In addition, the effect of INF-alpha was examined on rat hepatocytes undergoing oxidative stress and hepatic stellate cells (HSCs) in primary culture as well as isolated rat liver mitochondria (Lu *et al.*, 2002). INF-alpha activity led to dose-dependent increase of the immunoreactive protein levels of copper, zinc- and manganese-dependent SOD both in stressed hepatocytes and activated HSCs (Lu *et al.*, 2002). Kuwano *et al.* (2006) demonstrated that interferon- $\gamma$  activated transcription of NADPH oxidase 1 gene and upregulated (to 4-fold) production of superoxide anion by human large intestinal epithelial cells.

In plants, elevated SOD activity is related to enhanced tolerance to various stresses in term of larger plant biomass, which resistant cultivars or SOD

overexpressing plants can form in comparison with sensitive or wild-type ones (Jiang *et al.*, 2013; Sakhno, 2013). SOD gene over-expression correlates to biomass increase both in stressed and non-stressed conditions, in the laboratory as well as under field trials in alfalfa *Medicago sativa* L. (McKersie *et al.*, 1999), canola (Gusta *et al.*, 2009), rice *Oryza sativa* L. (Chen *et al.*, 2013), cotton *Gossypium barbadense* L. (Luo *et al.*, 2013). We proposed that exogenous INF application may influence plant SOD activity similarly to animal one. The aim of our study was to estimate the effect of recombinant human INF-alpha application on canola seed germination and seedling growth using fresh weight, total soluble protein content, and SOD activity evaluation.

### MATERIAL AND METHODS

#### Plant material and INF treatment

Seeds of spring canola (*Brassica napus* L.) cv Lega were surface sterilized, dried by filter paper and imbibed by water INF solutions with different concentrations ( $10^2$ ,  $10^3$ ,  $10^4$  IU/ml) for 30 min, 4°C. Seeds were kindly provided by Slisarchuk M.V. (Department of linseed and rapeseed selection and seed production of National Scientific center "Institute of UAAS on agriculture", Ukraine). There were three control treatments, namely 1) pure water, 2)  $10^2$  IU/ml INF inactivated by boiling, 3)  $10^2$  IU/ml INF with 2 mM DDC (SIGMA) as a SOD inhibitor (Heikkila *et al.*, 1976). Then seeds were dried by filter paper again and placed in Petri dish with the agar solidified MS medium (Murashige, Skoog, 1962) without hormones. Ten seeds were incubated in each Petri dish. Germination and seedling growth took place in thermostat at 24°C. Laferon (recombinant human interferon alpha 2b, 1 MIU, INTER-PHARM-BIOTEK, Ukraine) was used for INF solution preparation. Fresh weight (FW) of total seedlings, cotyledons, hypocotyls and roots was measured using the scale Pioneer™ PA413C (Ohaus Corporation, USA).

#### The total soluble protein (TSP) content

TSP content was determined using the Bradford method (Bradford, 1976). The extracts from plant leaves were prepared in triple volume of 100 mM Tris/HCl buffer, pH 8.0. The optical density was detected at 595 nm by BioPhotometer Eppendorf, v.1.35 (Germany).

#### SOD activity

SOD activity was measured using photochemical oxidation of nitro blue tetrazolium method (Beyer, Fridovich, 1987). Fresh plant material (100 mg) was

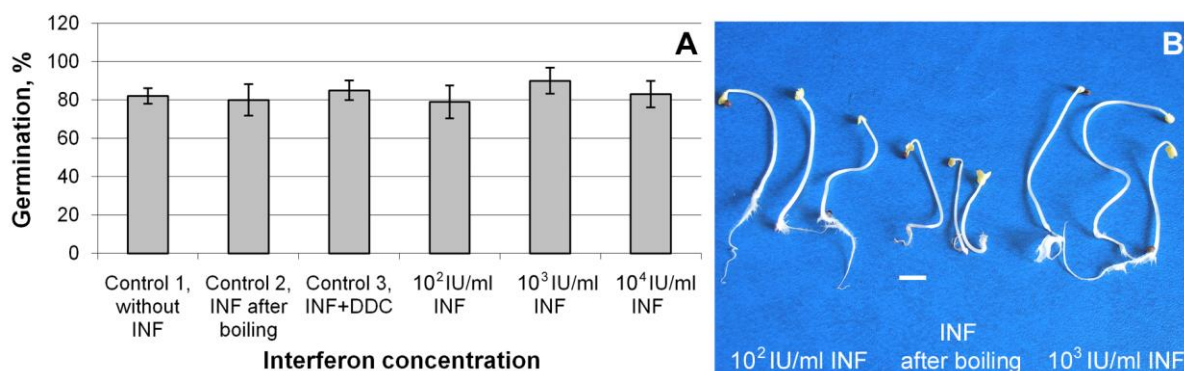
pounded with 1 ml of Tris-HCl buffer (pH 8.0) in a mortar and centrifuged at 13000 g (4°C) for 15 min. The supernatant was used for analyses. Formazan formation was held in a Eppendorf tube (1.5 ml). Plant extracts could inhibit this reaction due to SOD activity. One tube for each probe was retained in the dark. The others were illuminated with white light lamp (fluorescent lamp T5/G5, model ELI-230A-T5-8W) during 5 min in the thermostat at 24°C. Null probe had no leaf extract in its composition. In this probe oxidation was complete. The optical density of illuminated probe was measured at 550 nm by BioPhotometer Eppendorf (Germany) versus the optical density of dark probe.

### Statistical analysis

Statistical analysis was performed according to Duncan multiple range test. Differences from control values were significant at  $p \leq 0.05$ . Three independent experiments were conducted in five replications. One Petri dish with ten seeds was used as replication. There were nine replications for formazan measurement.

### RESULTS AND DISCUSSION

Canola seed germination in dark (thermostat, 24°C) conditions started two days after seed treatment. No differences in germination were detected between controls and INF treated seeds (Fig. 1, A). Seedlings which obtained from INF treated seeds had higher biomass in comparison with control ones (Fig.1, B, Fig.2, A).



**Figure 1** Canola cv Lega seed germination (A); 7-day-old seedlings were grown on agar solidified hormone free MS medium in thermostat at 24°C after seed treatment by water solutions with 10<sup>2</sup> IU/ml INF (left), INF inactivated by boiling (middle), and 10<sup>3</sup> IU/ml INF, (right) (B). Error bars represent mean±one standard deviation, scale bar: 1 cm

In seven-day-old seedlings, total FW and FW of cotyledons, hypocotyls and roots were measured and these parameters for single seedling were calculated (Fig.2, A). The results showed that application of interferon at concentration 10<sup>2</sup>, 10<sup>3</sup> and 10<sup>4</sup> IU/ml to canola seeds stimulated biomass accumulation in seedlings up to 2, 2.4 and 1.7-fold, respectively, compared with the control. Higher FW was measured in hypocotyls and roots; but not in cotyledons.

In case of total soluble proteins content, the differences in treated and control seeds were not reported for cotyledons; but the hypocotyls of treated seeds showed 2 times lower TSP content than control seedlings (Fig.2, B). Seed germination was accompanied by decrease in TSP content in seedlings of pea (*Pisum sativum* L.) (Diaz-Vivancos *et al.*, 2010), canola (Zielinski *et al.*, 2007, Sakhno, 2011), and radish (*Raphanus sativus* L.) (Zielinski *et al.*, 2007) during the progression of plant growth.

SOD activity was significantly lower in control seedlings (both cotyledons and hypocotyls) in comparison with INF treated ones (Fig.2, C). Moreover, the highest SOD activity was detected in seedlings from 10<sup>3</sup> IU/ml INF treated seeds. It elevated in cotyledons and hypocotyls 2.18- and 1.47-fold, respectively, above the SOD activity in controls. Rise in SOD activity was detected during non-stressed growth of pea, canola, and radish seedlings (Diaz-Vivancos *et al.*, 2010, Zielinski *et al.*, 2007). Time-dependent increase in SOD activity was shown in pea seedlings and plants, which could suggest an increased production of superoxide radicals (Diaz-Vivancos *et al.*, 2010). Shorning *et al.* (2000) demonstrated that the growth by elongation in wheat seedlings was controlled by the production rate of superoxide radicals. It was strongly suppressed under conditions of significant inhibition of the formation of superoxide radicals by synthetic antioxidant ionol. It can be assumed that production rate of superoxide radicals was higher in INF-treated canola seeds comparing to controls. It led to increase in the seedling growth by elongation and was accompanied by augment in SOD activity.

Previously it was shown that plant growth could be affected by application of various substances, of both plant and animal origin. Thus, the growth of *Arabidopsis thaliana* L. seedlings was promoted by progesterone (mammalian gonadal hormone) at low concentrations, but suppressed at higher concentrations under both light and dark growth conditions (Iino *et al.*, 2007). The growth of the gibberellin-deficient mutant of pea (*Pisum sativum* L.) was also promoted by progesterone (Iino *et al.*, 2007). TSP content as well as shoot and root length were increased by 16%, 12%, and 25%, respectively, in 25 day-old chickpea (*Cicer arietinum* L.) plants which were treated by 10<sup>-6</sup> M progesterone (Erdal, Dumlupinar, 2011). Growth improvement was accompanied by increase in activity of antioxidant enzymes including SOD (up to 1.14-fold). Exogenous application of other mammalian sex hormones (β-estradiol and androsterone) caused similar effects (Erdal, Dumlupinar, 2011)

Cytochrome P450<sub>SCC</sub> catalyzes three steps cholesterol oxidation with the formation of pregnenolone (precursor of progesterone) in animals (Chung *et al.*, 1986). Canola plants expressing bovine *cyp11A1* gene, which encodes cytochrome P450<sub>SCC</sub>, had higher leaf TSP content and increased SOD activity than wild-type ones in non-stressed conditions (Sakhno *et al.*, 2010, Sakhno, Slyvets, 2014). Accumulated pregnenolone tobacco plants with *cyp11A1* transgene (Spivak *et al.*, 2010) formed larger biomass and seeds in comparison with controls (wild-type and transgenic plants bearing only selective *bar* gene), but SOD activity was not evaluated (Spivak *et al.*, 2009).

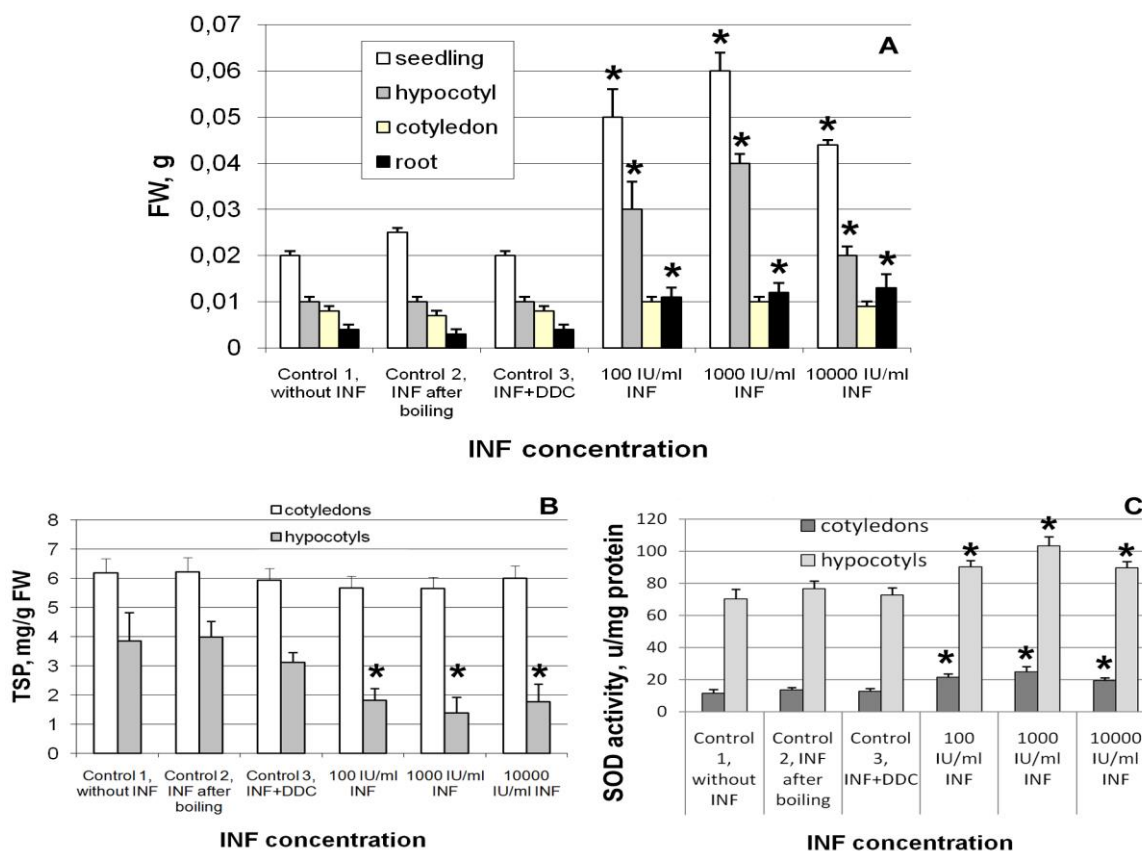
Exogenous melatonin treatment of wild type rice as well as its accumulation due to sheep serotonin N-acetyltransferase overexpression in transgenic plants enhanced root growth by seminal root elongation (Park, Back, 2012). Under non-stressed conditions 10% SOD activity increment was detected in transgenic rice in comparison with wild-type plants. The melatonin-treated maize (*Zea mays* L.) plants produced up to 20% more corn than controls (Tan *et al.*, 2012).

Expression of some heterologous genes in plants resulted in SOD activity increase and FW enlargement in non-stressed conditions. Thus, pine cytosolic glutamine synthetase (GS1a) enhanced growth of hybrid transgenic poplar (*Populus tremula x alba*) (Molina-Rueda *et al.*, 2013) with simultaneous SOD gene upregulation. Ectopic expression of riboflavin-binding protein *TsRfBP* gene from the soft-shelled turtle *Trionyx sinensis japonicus* led to SOD activity rise and FW growth in *Arabidopsis* plants (Deng, Dong, 2013). The same effect concerning to SOD activity was observed in non-stressed transgenic canola (Sakhno, Slyvets, 2014) and chicory (*Cichorium intybus* L.) (Kvasko, Matvieieva, 2013) plants expressing *huINFα2b* gene. It implies that substances that positively affect SOD activity have a positive impact on plant growth. Our experiments with human recombinant INF-α2b that was applied to canola seeds confirmed this fact.

Exogenous INF-α application is accompanied by superoxide radical formation and augment of SOD activity in animal cells (Al-Shabanah *et al.*, 1999, Pottathil *et al.*, 1981, Lu *et al.*, 2002). In present study rise in SOD activity was detected in hypocotyls and cotyledons of canola seedlings obtained from seeds treated by INF solution compared with controls. Increase in SOD activity was correlated to FW increment. NADPH oxidases are responsible for superoxide generation (Babior *et al.*, 1973, Keller *et al.*, 1998). The classical NADPH oxidase was first described and characterized in phagocytes, such as neutrophils, and it was originally thought that the enzyme was restricted to leucocytes and used solely for host defence (Babior *et al.*, 1973). Kuwano *et al.* (2006) demonstrated that interferon-γ activated transcription of NADPH oxidase 1 gene and upregulated (to 4-fold) production of superoxide anion by human large intestinal epithelial cells. A plant homolog of this oxidase was reported (Keller *et al.*, 1998) and intensively studied last years (Foreman *et al.*, 2003, Király *et al.*, 2008, Marino *et al.*, 2012). One of the transcripts suppressed in

knockdown *Arabidopsis* plants with suppressed expression of the thylakoid-attached Cu/ZnSOD was a transcript encoding NADPH oxidase (Rizhsky et al., 2003). These plants were suppressed in their growth and development. INF treatment may impact on plant biomass increase via stimulation of NADPH oxidase and SOD activities. Additionally, interferon as signal molecule could influence plant growth by interferon-induced proteins. One of them is the human double stranded RNA-dependent protein kinase (PKR) (Edelman et al., 1987). A

plant-encoded analog of PKR was identified in tobacco (*Nicotiana tabacum* L.), wheat and barley (*Hordeum vulgare* L.) (Crum et al., 1988, Langland et al., 1995). Multiple resistances to different plant RNA viruses such as cucumber mosaic virus, tobacco etch virus, or potato virus Y in PKR transgenic tobacco plants was proved (Lim et al., 2002). Further studies are needed for INF influence on plant growth.



**Figure 2** Physiological and biochemical parameters of canola seedlings after seed INF imbibition (growth in thermostat at 24°C): **A** - fresh weight of single 7-day-old seedling, **B** - cotyledon and hypocotyl total soluble protein content, **C** - SOD activity in cotyledons and hypocotyls. Error bars represent mean±one standard deviation and asterisk \* indicates significant differences between experimental values compared with control ones (p≤0.05)

**CONCLUSION**

Human recombinant INF-alpha 2b application on canola seeds caused an increase of biomass (up to 2.4-fold) in seven-day-old seedlings cultivated *in vitro*. In addition, positive correlation between the fresh weight increase of seedlings and SOD activity was reported. We suppose increased SOD activity which occurred due to seed imbibitions by interferon solution improved plant biomass.

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