EFFECT OF SOME THERAPIES ON POTATO PLANTLETS INFECTED WITH POTATO VIRUS X (PVX)

C. L. BĂ D Ă RĂU[**] **N. CHIRU**[***]

Abstract: *The purpose of this study is to decrease the PVX (potato virus X) infection level, using electrotherapies, antiviral compounds (ribavirin and oseltamivir) in the tissue culture and several treatments (Satureja hortensis essential oils, H_2O_2 1mM pH 5.6) applied to microplants acclimatisated in green house. The biological material used in experiments was plants (variety Roclas, virus free biological material) mechanically inoculated using: PVX secondary infected plants from Bintje variety. Electrotherapy was applied in 9 variants: after washing and sizing explants, potato stems infected were exposed to either 40, 50 or 100 miliampers (mA), for 5, 10 or 20 minutes, followed by sterilization and immediate planting the axillary buds tip in vitro. Chemotherapy was undertaken with ribavirin (RBV) and oseltamivir (OSMV) (RBV 40 mg l^{-1} +OSMV 40mg l^{-1} ; RBV 20mg l^{-1} + OSMV 40 mg l^{-1}; RBV 20mg l^{-1} + OSMV 80mg l^{-1}). The first variant (RBV40mg l^{-1} + OSMV40mg l^{-1} added to the tissue culture medium + essential oils treatments of acclimatisated plants) and the electrotherapy variant 10minutes at 100mA showed the highest rate of virus eradication, the maximum values of the therapy efficiency.*
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Keywords: *Satureja hortensis essential oils, potato virus X, Ribavirin, Oseltamivir, electrotherapy*

1. Introduction

Potato virus X (PVX), a *Potexvirus*, occurs throughout commercial stocks of most varieties and is responsible for many of the uncertainties and difficulties encountered in field inspections. When *potato virus Y* is present, synergy between these two viruses causes severe symptoms in potatoes.

Elimination of PVX from potato supply is essential for seed potato production. Also, in this study, the efficiency of some techniques (chemotherapy, treatments with *Satureja hortensis* oils, electrotherapy) in eliminating PVX and producing virus-free plants (cultivar Roclas) was evaluated.

Untill now, many compounds were tested for their antiviral activity but few were effective (Schuster, 1988). The most used substance is the ribavirine (Virazol), an analogue of guanosine, wich when added to the medium at concentrations of 10-50mg/l, was effective against PVX, PVY, PVS and PVM in potato (Cassel and Long, 1982, Klein and Livingston, 1982; 1983; Cassel, 1987; Griffiths 1990). However, ribavirin at active dose is usually phytotoxic causing an increase in culture time, death of some meristems, and the need for frequent transfers to fresh media (Klein and Livingston, 1982). Regeneration of potato sprouts from meristematic cultures was delayed by 6 to 8 weeks (Cassel, 1987). To overcome toxicity, a low dose of ribavirine was supplied with another compounds (antimethabolites).The simultan use of the two chemicals alone was also beneficial, because ribavirin above 5 mg/l delayed meristem development. In our research we used oseltamivir (Tamiflu) for reducing the phytotoxic effect of the ribavirine.

The treatments with *Satureja hortensis* essential oils and antioxidants (H_2O_2 and ascorbic acid) applied to acclimatised plants (obtained planting the plantlets) could be beneficial for obtaining virus free material. The essential oils from *Satureja hortensis* L. (summer savory – Family *Lamiaceae*, order *Lamiales*) are known for its antiseptic (antifungal and antiviral) properties (Bedoux et al., 2010). Maybe some compounds ot these oils could be implicated in the processus signaling against stress, in infected potato plants (Bădărău, 2012; Bedoux, 2010).

The methods employed to eliminate viruses from plants like meristem culture, chemotherapy and thermotherapy are technically demanding and time consuming. Electrotherapy, however, is a simple method of virus eradication without the

** Dept. IMAT,Faculty of Food and Tourism, T*ransilvania* University of Braşov, Romania, carmen.badarau@unitbv.ro
*** National Institute of Research & Development for Potato and Sugar Beet Brasov, e-mail: carmenbadarau@potato.ro

need to use any special or expensive equipment. In this technique, the electric current is applied to plant tissues in order to disrupt or degrade viral nucleoprotein and eliminate its virulence activity (Lozoya-Saldaña et al., 1996, Sabry et al., 2009, Hormozi-Nejad et al., 2010).

The study aimed to evaluate the therapy efficiency of different chemotherapies and treatments with *Satureja h.* EOs applied to acclimatised plants and several electrotherapies, for PVX elimination in potato plants and find out the best one for virus eradication.

2. Material and method

Solanum tuberosum L. microplants cv. Roclas, tested virus free, were obtained from the Biotechnology Department of National Institute of Research and Development for Potato and Sugar Beet Brasov. The microplants were transferred to greenhouse conditions 30 days. For obtaining positive material, a part of these plants were mechanically inoculated (Bădărău et al., 2012) using a PVX secondary infected source cv. Ostara.

The plants had previously tested positive by ELISA for PVX, to confirm the occurrence of single infection in the selected material. Tissue samples infected mother plants growing in the greenhouse were used as positive control. Stem segments excised from infected potato plants were transferred two times in MS medium with antiviral compounds (sub-culture S1-26 days, sub-culture S2 -30 days). Plantlets obtained were divided into single node cuttings (about 1cm length) and sub-cultured on a fresh MS medium (sub-culture S3). After 28 days the plantlets were planted in pots, under greenhouse conditions.

Acclimatization, treatments with *Satureja hortensis* **essential oils** *Solanum tuberosum L.* plantlets submitted to chemotherapy, regenerated with roots and a well developed aerial part (5-7 leaflets), were removed from the culture medium and were acclimated in pots containing a sterilized mixture of soil, vermiculite and organic matter (2:2:1). After 7 after the injection with EOs suspension (14 days for beginning the acclimatisation), the plants (excepting the controls) were sprayed twice a week with a *Satureja hortensis* essential oils suspension (1/1000, 5 ml each plant) (Bădărău et al., 2012). The survivor plants were indexed after 45 days from the transfer in the green house.

DAS ELISA test. The analysis was performed following the protocol Clark and Adams (1977).

Chemotherapy was carried out on nodal cuttings with a single axillary bud and was undertaken with ribavirin (RBV, Sigma, Q0125) and oseltamivir (OSMV, Tamiflu, LaRoche) in the following variants: V1= RBV 20 mg l^{-1} + OSMV 40mg l^{-1} ; V2 = RBV 40mg l^{-1} + OSMV 40 mg l^{-1}; V3 = RBV 20mg l^{-1} + OSMV 80mg l^{-1}.

Table 1 Chemicals used for obtaining PVX virus-free plantlets

Chemicals	**Activities**	**Chemicals Activities References**
Ribavirin (Virazol) (1,ß-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide) (Abbreviation in text RBV)	Broad spectrum anti-viral activities, ribavirin 5'-phosphate: inhibitor of inosine monophosphate (IMP) dehydrogenase	Cassel and Long (1982)
Oseltamivir (Tamiflu) [ethyl (3R,4R,5S)-5-amino-4-acetamido-3-(pentan-3-yloxy)-cyclohex-1-ene-1-carboxylate] (Abbreviation in text OSMV)	- an antiviral prodrug - used to slow the spread of flu virus (influenza A and B) by stopping from chemically cutting with its host cell. - produced from shikimic acid, an inhibitor of neuraminidase	Ward et al. (2005)

Electrotherapy treatments and regeneration of virus-free plants. Before the treatment, the greenhouse-grown inoculated plants were assayed by DAS-ELISA for verify the virus presence. Plants with similar levels of virus concentration were used to obtain stem segments containing axillary buds for electrotherapy. Each infected plant provided for approximately 3 nodal cuttings that were subsequently used for electrotherapy treatment. From each stem one node was cut for the control (untreated by electrotherapy) and the stem segments remaining were immersed in natrium chloride solution (1M) in an electrophoresis tank and exposed to electric currents of 40, 50 and 100 mA for 5, 10 and 20 minutes using a power supply (Tehsys E250V,

fig. 1). After treatment, the stems were surface sterilized and rinsed three times in distilled water (fig. 1). Explants were prepared by dividing stem segments into nodal cuttings with a single axillary bud. The cuttings were cultured in test tubes containing MS medium (fig. 1). The experiment was repeated three times for each electrotherapy treatment.

In the aime to estimate an electrotherapy treatment leading to high rates of both virus elimination and plant regeneration, the Therapy Efficiency Index (TEI) was used (Lozoya-Saldaña et al. 1996). The TEI was estimated with the following relation:

TEI = percentage of regenerated plantlets × percentage of virus-free samples / 100

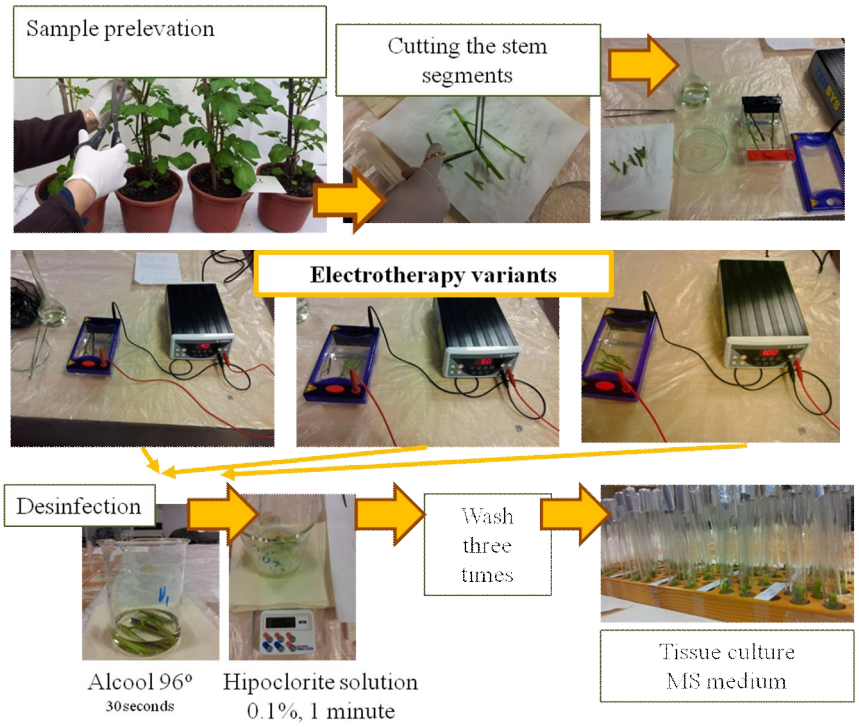
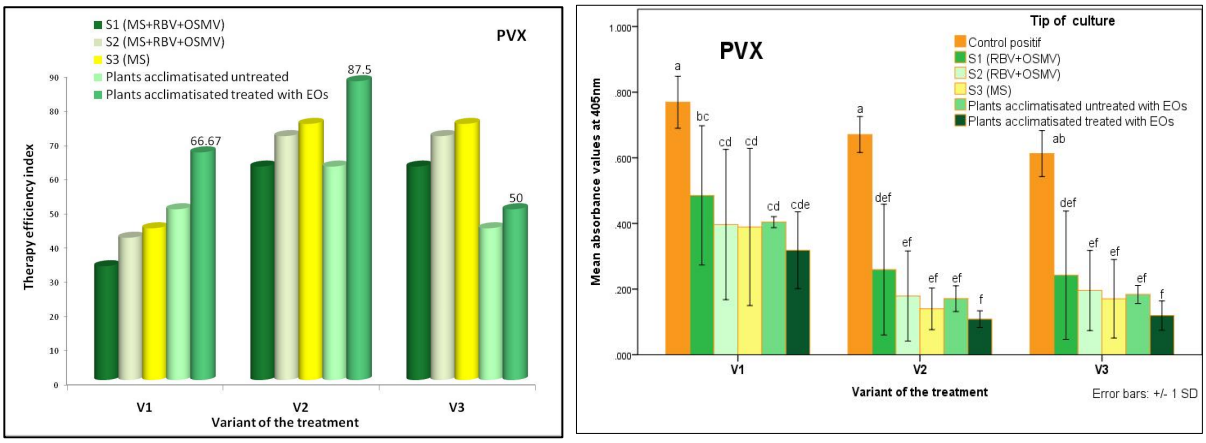


Fig.1. *Electrotherapy treatments steps and equipment used to produce virus-free plants in potato cv. Roclas: Power supply and electrophoresis tank used for producing electric currents, single node explants prepared for in vitro culture, electrotherapy variants (intensity of the electric current) regeneration of electrotherapy treated plants on MS medium.*

3. Results and discutions

Chemotherapy. Results showed that in all the variants and stage of the therapy, for both viruses, plants virus free were found (table 2).

Regarding the PVX infected plants, the chemotherapy variant V2 (RBV 40mg/l + OSMV 40mg/l) combined with treatments with *EOs* and AO of acclimatisated plants, lead to the higher value for the virus elimination rate (100% PVX free plants) and for the therapy efficiency index (TEI): 87.5%. (table 2; fig. 2). Highest values for the virus elimination rate (100%) were obtained in variant V3 (RBV 20mg/l + OSMV 80mg/l) combined with treatments *EOs* +AO too, but this treatments decreased regeneration rate (50%), also the TEI had lower values than in variant V2 (table 2; fig. 2).The absorbances values at 405nm (DAS ELISA) for the plants acclimatized obtained from the variant V2 (RBV 40mg/l + OSMV 40mg/l) and treated wit EOs and AO were significantly lower (fig. 2).



A. **B.**

¶ (5…10 mm)

Fig. 2. *Evaluation of chemotherapy and treatments with Satureja hortensis essential oils and H_2O_2 (1mM) on plants infected with potato virus X (PVX).The therapy efficiency of differents variants and vegetation culture (B) The average values of absorbance in the plants regenerated from infected plants inoculated with a PVX viral strains (from cv Bintje secondary infection) Data are means ± SD of 3 experiments (n=3). Bars with different letters differ significantly by Duncan's test (P<0.05).*

Table 2. Efficiency of chemotherapy, special treatments* of acclimatisated plants on PVX infected material regeneration and virus eradication (cv. Roclas)

Variant of the treatment (for potato virus X infected plants)		**Regeneration rate**		**Virus elimination rate**	
		NPT/NRP	%	NPVF/NPM	%
V1	S1 (MS + antivirals)	5/6	83.3	1/5	40
	S2 (MS + antivirals)	10/12	83.3	5/10	50
	S3 (MS)	16/18	88.9	8/16	***50.0***
	Plants acclimatisated untreated	6/6	100	3/6	50
	Plants acclimatisated treated *	5/6	**83.3**	3/5	**60**
V2	S1 (MS + antivirals)	7/8	87.5	5/7	71.4
	S2 (MS + antivirals)	12/14	85.7	10/12	83.3
	S3 (MS)	22/24	91.7	18/22	***81.8***
	Plants acclimatisated untreated	7/8	87.5	5/7	71.4
	Plants acclimatisated treated *	7/8	**87.5**	7/7	**100**
V3	S1 (MS + antivirals)	5/8	62.5	4/5	80
	S2 (MS + antivirals)	7/10	70.0	6/7	87.5
	S3 (MS)	10/16	62.5	9/10	***90***
	Plants acclimatisated untreated	4/6	66.7	3/4	66.67
	Plants acclimatisated treated *	3/6	**50.0**	3/3	**100**

V1= MS +RBV(20mg/L) + OSMV(40mg/L); V2 = MS +RBV(40mg/L) + OSMV(40mg/L);V3= MS +RBV(20mg/L) + OSMV(80mg/L); MS =Murashige and Skoog medium; RBV=Ribavirine; OSMV=Oseltamivir; NTP = number of tested plants (plants that survived); NRP = number regenerated plants; NPVF = number of plants virus free;
*Treatments with *Satureja hortensis* essential oils and H_2O_2 (1mM)

Electrotherapy

Application of electrotherapy on the potato cultivar Roclas resulted in partial elimination of PVX from potato tissues when the most severe treatments were applied (100 mA for 10-20 minutes). The figures 3,4 showed that the two viruses were not very different in responding to electrotherapy (excepting the variant 100mA, 10minutes). In spite of developing of many virus-free plants, diminishing levels of virus concentration were observed in all variants even if the regenerated plantlets remain infected, for both viruses (fig. 4). But the success of electrotherapy in producing virus-free plants depends upon both plant regeneration and virus elimination rates.Usually, plant regeneration depends upon several factors, including genotype, physiological state of the explant, culture medium, the cultivation conditions and the interactions between these factors (Svetleva et al. 2003). The electric pulses are also reported as stimulants of plant differentiation *in vitro* (Hormozi-Nejad et al., 2010). It was demonstrated that regeneration of potato plant tissues could be improved by exposing explants to mild electric currents (Lozoya-Saldaña et al. 1996).

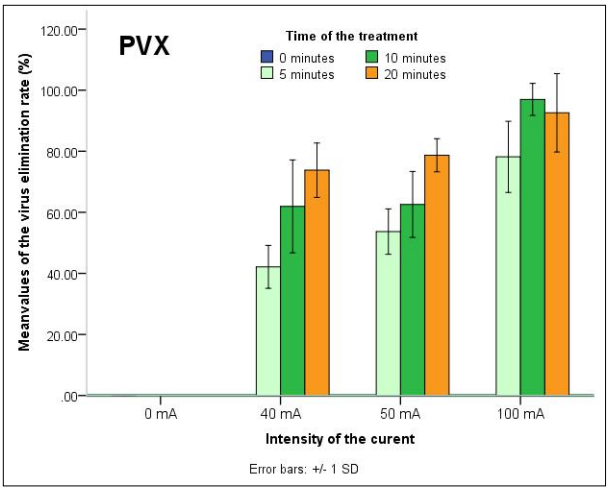


Fig. 3. *Effects of electrotherapy treatments on the elimination rate of PVX in Roclas cv. infected material. Results = the mean of three experiments.*

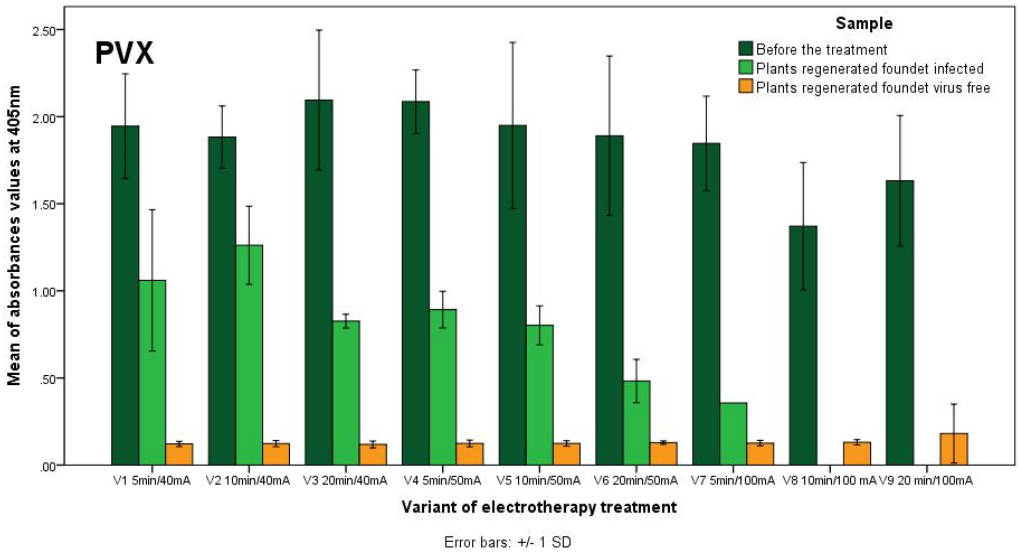


Fig. 4**.** *Mean absorbances values of regenerated plants, using different electrotherapy treatments on potato plants (cultivars Roclas): OD in infected plants before electrotherapy (green dark bars) and in regenerated plants after electrotherapy which were ELISA positive (green bars) and ELISA negative (orange bars). Results = the mean of three experiments.*

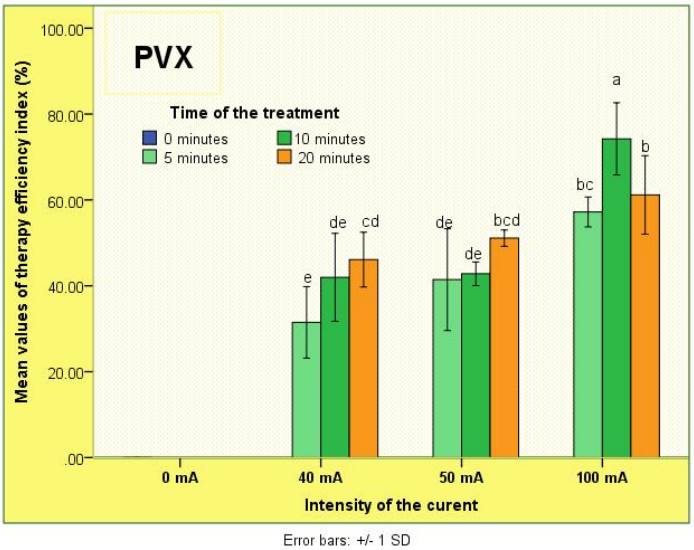


Fig. 5. *Effects of electrotherapy treatments on the Roclas cultivar infected explants. Mean of the therapy efficiency index (TEI) for PVX infected and treated material. Results = the mean of three experiments. Bars with different letters differ significantly by ANOVA and Duncan's test (P<0.05).*

Plant regeneration rate estimated as the ratio of the number of regenerated plantlets to the total number of cultured explants was 56.1- 77.2%. Usualy, higher intensities of electric current affected the survival of explants and thus plant regeneration.

The results of the present research work show that the regeneration of explants *in vitro* is influenced by electrotherapy treatment and depends upon the electric current intensity. Many papers suggest that the regeneration rate of virus-free plants obtained after electrotherapy is higher than that of plants exposed to more conventional virus elimination techniques including meristem culture and chemotherapy (Jung-Yoon et al. 2003; Mahmoud et al. 2009). In our study we obtained good results regarding the regeneration rate when the oseltamivir was used or when the acclimatisated plants were treated with *Satureja hortensis EOs* and AO.

Regenerated plantlets were tested for PVX infection by DAS-ELISA. A reduced concentration of virus was observed in all regenerated plantlets (fig. 4). On this basis, the mean virus elimination rates of Roclas cultivar explants exposed different periodes to the three electric currents of 40, 50 and 100mA was 62/7%, 54.9% and 88.7% for PVX infected material (figure 3). An increase in the number of virus-free plants for both viruses was observed as the intensity of the electric current was raised. The highest virus elimination rate was obtained at the highest electric current (100mA) used in this study. Although raising the electric current increased the mean virus elimination rate, it also decreased the mean plant regeneration rate, so TEI index has to be used as a basis in identifying the most efficient electrotherapy treatment. The TEI for the three electrotherapy treatments at 40, 50 and 100mA was estimated as 42.6, 44.6 and 64.3. The electric currents of 100 mA for 10 minutes resulted in the highest TEI value: 74.3 for PVX infected material (figure 5).

It has been postulated as a hypothesis that viral nucleoproteins may be denatured by when it is exposed to electric current (Lozoya-Saldaña et al. 1996). It has been suggested that denaturation of viral particles may occur during transport through the plasmodesmata in the apoplastic space. Inactivation of specific nucleoprotein that assist in cell-to-cell movement to threedimensional structures leads to blockage, which prevents further penetration of virus particles to healthy cells (Hormozi-Nejad et al., 2010; Hull R., 2002). The basis of this phenomen is still poorly understood.

4. Coclusions

This preliminary study revealed that chemotherapy (40mg/l RBV + 40mg/l OSMV), followed by treatments with *Satureja hortensis* EOs and AO of acclimatisated plants on the one hand and the electrotherapy (100mA, 10 minutes) on the another hand, had effects on PVX elimination from potato plant tissues.

Further investigations are needed for combine chemotherapy + electrotherapy + treatments with *EOs* and AO of the

acclimatisated plants, for improvement and optimization of these techniques.

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Lecturer Cristina Canja Ph. D., Transilvania University of Brasov, Romania

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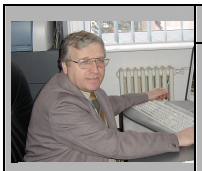

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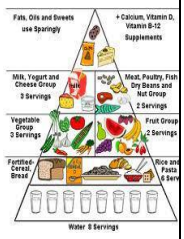

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