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# Sodium Dichloroisocyanurate: An eco-friendly chemical alternative for media autoclaving and explant sterilisation in plant tissue culture

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Abstract

Autoclaving nutrient media is still considered as the optimum mode of sterilisation in plant cell and tissue culture. During the process steam under high pressure is maintained at 120 degrees Celsius, 15 psi for 15-20 minutes in a chamber, optimised to kill all possible microbial life forms. But the disadvantages related to the process of autoclaving are plentiful. They are, decrease in the media pH, salt precipitation, agar depolymerisation, carbohydrate hydrolysis, volatile obliteration and necessity of the infrastructure investment. Requirements of additional resources (time, human resources, electrical energy) have forced the lookout for a more viable alternative, that is, chemical sterilisation. The use of Sodium dichloroisocyanurate (NaDCC) is a useful alternative for media and explant sterilisation. NaDCC is stable, water-soluble, non-toxic and easy to use at room temperature, does not have any environmental hazards and is not phytotoxic. The use of NaDCC as a disinfectant has been documented well concerning water sterilisation, surface sterilisation and also as a broad spectrum disinfecting agent. Disinfecting property of NaDCC is due to the hydrolytic release of chlorine, and this can be utilised for sterilisation of media and explants in plant tissue culture. NaDCC is a useful alternative for autoclaving at a concentration range of 0.05 to 1.0 g/l. However, only a few reports are available for its use as a sterilising agent for media and explants for *in vitro* cultures of plants. This paper discusses and reviews the possibility of establishing NaDCC as an active agent for explant sterilisation and as a viable alternative to medium sterilisation through autoclaving.

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## INTRODUCTION

*In vitro* culture of plant cells and tissues is one of the most important tools utilised in plant science research. The success or failure of *in vitro* culture depends on the maintenance of aseptic cultures (Espinosa-Leal *et al.*, 2018). The current practice for sterilisation of plant tissue culture media and glassware is to autoclave at a sterilisation temperature of 121$^0$C at a pressure of 1.05 -1.40 kg/cm$^2$ (15-20 psi) for a minimum duration of 15 minutes. However, the period of sterilisation time depends on the volume of media per vessel (Burger, 1988). In addition to autoclaving, substances such as antibiotics have to be added to the media to prevent contamination (Falkiner, 1997). The main demerits of autoclaving are (i) huge costs on electricity (Chen, 2016) (ii) thermal destruction of compounds (Schenk *et al.*, 1991) and (iii) moisture retention which attracts contamination after autoclaving (Pullaiah *et al.*,

2017). Plant tissue culture techniques are considered as a productive small scale enterprise (Mascarenhas, 1999; Rajmohan, 2011), especially for the upliftment of rural places and rural women. It has also been recorded that there is a higher participation of women in the plant tissue culture industry (Reddy, 2007). However, substantial electricity cost is one of the critical factors that negate the popularisation of plant tissue culture-based enterprise (Pożoga *et al.*, 2019). Plant tissue culture has the potential to be a catalyst for the development in both the agricultural and industrial sectors in the emerging economy (Aladele *et al.*, 2012). Therefore a viable chemical method of sterilisation is the demand of the time for successful implementation and economic feasibility.

Chemical sterilisation of explants in plant tissue culture is the most important process *in vitro* cultures. Traditionally mercuric chloride is used as a chemical disinfectant for explants. However, mercuric chloride is highly toxic and environmentally hazardous. It also imparts health threats at the user end. So scientists are in constant search for an effective, eco-friendly alternate option for mercuric chloride sterilisation. Sodium Dichloroisocynaurate ($C_3Cl_2N_3NaO_3$; 1,3-dichloro-1,3,5- triazinane-2,4,6-trione, Dichloroisocyanuric acid, sodium salt- Figure 1) is a water-soluble white crystalline powder with bleach-like odor. Chemically NaDCC is a chlorinated hydroxytriazine salt (USCG, 1999). This review paper is to examine the possibilities of NaDCC as an alternate, cost-effective, environment-friendly option as a sterilant for explants as well as media in plant tissue culture.

**Mechanism of Action**

The compound in the presence of water releases free chlorine as hypochlorous acid through the following hydrolysis reaction (Kuznesof, 2003; Phong *et al.*, 2018).

$$C_3N_3O_3Cl_2Na + 2H_2O \leftrightarrow C_3N_3O_3H_2Na\ (sodium\ cyanurate) + 2HOCl\ (hypochlorous\ acid)$$

Since NaDCC releases free available chlorine, it is widely used as a disinfectant in the swimming pool, food industry and cleaning surfaces (WHO, 2004). Chlorine induces leakage of cell contents because it alters the permeability of the cell membrane leading to the death of bacteria (Venkobachar *et al.*, 1977). Sodium cyanurate, the product released in the reaction doesn't exhibit evident toxic effects (Hodge *et al.*, 1965). NaDCC has been approved as an agent for routine and emergency treatment of water by the United States Environmental Protection Agency and the WHO respectively. It is an ideal disinfectant because of the stability, credibility and cost-effectiveness the compound offers upon its usage (Clasen and Edmondson, 2006).

NaDCC tablets, as well as the solution, has a sufficient reserve of available chlorine, strip packaged NaDCC, and has a shelf life of five years in tropical and temperate climates (Clasen and Edmondson, 2006). Hence it retains its stability and the ability to be reused over extended periods in the lyophilised form, making it exceptionally economically convenient. NaDCC dissociates into cyanurate and hypochlorous acid when dissolved in water. There is partial storage of chlorine in the form of chlorinated isocyanurates and partial expelling of it as free available chlorine. When the balance of the system involving this compound gets compromised due to either pH changes or exposure to high organic loads, this ability of the mixture makes it more efficient than other chlorine compounds that use hypochlorous acid as a disinfecting agent.

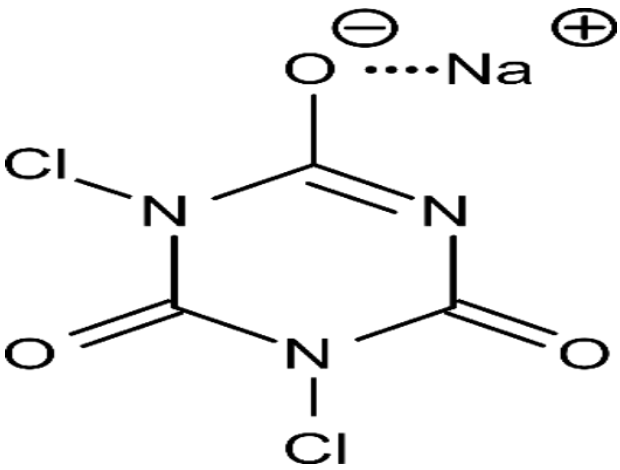


**Figure 1: Sodium Dichloroisocynaurate**

The NaDCC is easily hydrolysed when added to the water. Six chlorinated and four non- chlorinated isocyanurates create the equilibria (Kuznesof, 2003). the quantities of NaDCC used should be the lowest compatible with proper disinfection. It is also crucial that cyanuric acid concentrations should be kept as small. Sodium cyanurate is readily degraded in the environment and forms carbon dioxide and ammonia.

Therefore NaDCC or its products does not accumulate in the environment. Therefore NaDCC ensures disposal safety. Hence NaDCC has an advantage over mercuric chloride used for sterilisation which is extremely hazardous to the environment. It has been found via preclinical trials that chlorine doses even up to a high dose of 10 mg/l are not detrimental to health. The safe level of sodium cyanurate concentration is below 11 mg/l (WHO, 2004).

**Toxicity**

NaDCC does not have any significant toxicity towards the cultures of micropropagation, and it has been successful in maintaining the growth rate, quality of shoots during shoot culturing and disinfection of more than fifty plant species (Parkinson *et al.*, 1996). NaDCC has a slow rate of biodegradation, but the products produced at the end of it, like, ammonia, cyanuric acid, and carbon dioxide are incredibly beneficial for the environment. These can act as nitrogen sources for fixation by bacteria in soil, sewage, river water, and activated sludge. The performance of NaDCC with the introduction of organic load in a system depends on various factors such as pH, nature of the chlorine compound and the nitrogen: chlorine ratio (Bloomfield and Uso, 1985). NaDCC works as an inert sterilising agent, satisfying the requirement of an excellent disinfectant of nutrient media in plant tissue culture.

NaDCC solutions are acidic. Depending on the pH, the free available chlorine exists either as hypochlorous acid or hypochlorite ion. The ratio of hypochlorous acid to hypochlorite ion increases steeply with a decrease in pH. This acts as the predominant active species required to bring about the necessary bactericidal action (Coates, 1988). This property makes the compound a more obvious choice in comparison to the other chemical compounds like sodium and calcium hypochlorite. NaDCC tablets may require less cost to optimise utilisation. Thus it is possible to be more cost-effective even if its actual price as a commodity might be higher.

The chemical compound has a robust sporicidal activity and counteraction against vegetative bacteria, in a fixed pH range when applied with sodium hydroxide (Bloomfield and Arthur, 1992). It is not only effective against *Enterococcus faecalis, Streptococcus salivarius, Streptococcus sobrinus* and *Streptococcus mutans* at an optimised minimum inhibitory concentration but also has even shown sufficient cytotoxic activity, almost at par with sodium hypochlorite in all the experiments conducted to test its efficacy (Heling *et al.*, 2001). In addition to several bacterial and fungal species (Table 1), viruses such as adenovirus, calicivirus, hepatitis A virus, poliovirus and rotavirus and protozoans like *Entamoeba histolytica* and *Giardia lamblia* (Clasen and Edmondson, 2006) are vulnerable to the action of hypochlorous acid. The efficient maintenance and application of hypochlorous acid in NaDCC to stabilise the disturbance in intrinsic variables (pH and temperature), makes it a more reliable disinfecting alternative to be used in situations involving these microbes. It has surpassed its chemical alternatives in its action against aerobic mesophiles, moulds, yeasts and many other bacteria such as *E coli* and *Salmonella* when used as a sterilising agent for freshly grown vegetables (Clasen and Edmondson, 2006). The combined effect of Gamma irradiation and NaDCC has highly effective postharvest fungal resistance. NaDCC was also declared as a potential eco-friendly fungicide in fruits (Jeong *et al.*, 2015).

NaDCC inhibits a wide range of gram-positive and gram-negative bacteria as well as fungi (Table 1). However, NaDCC does not show any teratogenic, mutagenic or carcinogenic side effects making it a safe disinfectant. NaDCC has many advantages associated with its usages such as (i) A long shelf life of five years, (ii) Resistant to sunlight-induced chemical degradation (iii) Ideal for single-use packaging (iv) feasibility of low weight in distribution (Lantagne *et al.*, 2010).

**Potential advantages of NaDCC in plant tissue culture**

The overall idea is to bypass autoclaving, which is a time-consuming procedure apart from the cost of electricity, infrastructure availability and human expertise. Therefore, it is logical to use a chemical that has significantly less or negligible environmental toxicity to prevent contaminating microorganisms in tissue culture media (da Costa Urtiga *et al.*, 2019). The use of such an agent will also reduce the cost of plant tissue culture enterprise. NaDCC is having all the ideal properties, including stability, low toxicity and ease of handling (da Costa Urtiga *et al.*, 2019). The potential application of chlorine disinfectants in plant tissue culture sterilisation was first experimented by Yanagawa *et al.* (2007) for micropropagation of ornaments such as *Cymbidium* plantlets and *Phalaenopsis* plantlets through direct regeneration. Media was not autoclaved, and inoculation was performed without laminar airflow. No phytotoxic effect was observed in these cases. The most effective concentration range was from 0.05 to 1.0 g/l.

Further, the use of NaDCC at 0.02 g/l has been linked to enhanced seed germination of *Dianthus caryophyllus* under *in vitro* conditions. This fact is further evidence of reduced or no phytotoxic effect. In any case, the contamination rate has never been over 5% (da Costa Urtiga *et al.*, 2019).

Another use of NaDCC is as a chemical sterilising agent for explants. NaDCC was effective against *Pseudomonas*, *Xanthomonas*, and Actinomycetes. Samples were analysed for the efficacy of NaDCC with explants that were heavily contaminated with

**Table 1: Antibacterial and antifungal activity of Sodium Dichloroisocyanurate**

| Bacteria | References |
|---|---|
| **Gram-positive** | |
| *Bacillus sp* | (Coates, 1996) |
| *Enterococcus faecalis* | (Heling *et al.*, 2001) |
| *Lactobacillus sp* | (Guiteras and Schmelkes, 1934) |
| *Staphylococcus sp* | (Bloomfield and Uso, 1985; Proto *et al.*, 2016) |
| *Streptococcus mutans* | (Heling *et al.*, 2001) |
| *Streptococcus salivarius* | (Heling *et al.*, 2001) |
| *Streptococcus sobrinus* | (Heling *et al.*, 2001) |
| **Gram-negative** | |
| *Acinetobacter sp* | (Abreu *et al.*, 2013) |
| *Campylobacter jejuni* | (Clasen and Edmondson, 2006) |
| *Enterobacteria sp* | (Abreu *et al.*, 2013) |
| *Escherichia coli* | (Clasen and Edmondson, 2006; Proto *et al.*, 2016) |
| *Pseudomonas aeruginosa* | (Lantagne *et al.*, 2010) |
| *Pseudomonas sp* | (D'Auria *et al.*, 1989) |
| *Salmonella dysenteriae* | (Clasen and Edmondson, 2006). |
| *Vibrio cholerae* | (Lantagne *et al.*, 2010) |
| *Yersinia enterocolitica* | (Clasen and Edmondson, 2006) |
| **Fungus** | |
| *Aspergillus sp* | (Proto *et al.*, 2016; Staniszewska, 2004). |
| *Botrytis cinerea* | (Jeong *et al.*, 2015) |
| *Microsporidia* | (Zawawy *et al.*, 2010) |
| *Penicillium sp* | (Jeong *et al.*, 2015) |
| *Scopulariopsis sp* | (Bundgaard-Nielsen and Nielsen, 1996) |
| Yeasts | (Bundgaard-Nielsen and Nielsen, 1996) |

bacteria. The experiments revealed that NaDCC was equally effective as mercuric chloride. Even though the ideal concentration was 5000 ppm, a lower concentration up to 300 ppm is also found to be useful.

Further, it was confirmed that NaDCC is stable at room temperature (Parkinson *et al.*, 1996). A study conducted by Mihaljević *et al.* (2013) reported that the use of NaDCC at a concentration of 1% for 20 minutes was not satisfactory compared to similar levels of other sterilising agents such as silver nitrate, mercuric chloride, sodium hypochlorite, and hydrogen peroxide. According to a treatment of 300 ppm, sodium dichloroisocyanurate for 48 h is beneficial in sterilising explants from the greenhouse and field-grown plants. Caton (2008) also developed a successful protocol for the sterilisation of woody plants with NaDCC. Rowntree and Ramsay (2005) used a 1% solution of NaDCC for 4-6 minutes in sterilising bryophyte explants for *in vitro* cultures (Yanagawa *et al.*, 2007). NaDCC is a broad spectrum disinfectant limiting the growth of fungi and bacteria (Table 1). Many of these are also frequent contaminants of plant tissue culture (Leifert *et al.*, 1989; Cassells, 1997; Cobrado and Fernandez, 2016). Therefore it is logical and feasible to use NaDCC for the sterilisation of media as well as explants.

## CONCLUSION

NaDCC has demonstrated very promising results, but in extremely controlled and optimised set up, as a nutrient medium sterilant in plant cell and tissue culture. NaDCC should be tested in longer, randomised controlled trials against already existing disinfecting agents, and over many varieties of plant cultures. This would be extremely useful in establishing its benefits and applicability as a better alternative to autoclaving or at least as effective as the same. NaDCC has been proved to be less effective than sodium hypochlorite in terms of its action against bacterial spores, the influence of the intrinsic parameters on the result has a potential scope for detail. The potential effects of NaDCC over the metabolism of plantlets cultured in the nutrient media sterilised by the same also lacks experimental proof. It's potential as an explant sterilant or a glass-

ware and tools sterilant in plant tissue culture has also not been looked into. In terms of being utilised as a water treatment agent, the inconsistent data obtained to establish its potential impact on health, bioaccumulation, and formation of trihalomethanes and cost-effectiveness has not been addressed substantially. Manipulation of parameters and development of model systems for prolonging the shelf life of plant cultures *in vitro* using NaDCC should be experimentally carried out as there is a cornucopia of theoretical knowledge yet to be proved.

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**Conflict of Interest**

The authors declare that there is no conflict of interest for this study.

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