ATMOSPHERIC COLD PLASMA (ACP) TREATMENT FOR EFFICIENT DISINFESTATION OF COWPEA WEEVILS *(CALLOSOBRUCHUS MACULATUS)*

A Thesis

by

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ABSTRACT

Understanding and preventing post-harvest losses are very crucial if we want to feed the world's growing population. *Callosobruchus maculatus*, also known as the "cowpea weevil," is a common pest in stored grains. This pest attacks stored grains, causing grain weight loss, nutritional value decline, quality deterioration, and seed viability and vigor to decline. Atmospheric cold plasma (ACP) is a novel treatment technology that produces an ionized gas containing reactive oxygen and nitrogen species, electrons, and free radicals, all of which are capable of killing microorganisms. It has the potential to disinfect stored grains and be a safer alternative to existing pesticides. The possibilities of ACP as a feasible pesticidal alternative were explored in this study. Using ionized gas from the ACP treatment system we see mortality rates of 82–92%, demonstrating that ACP is a viable treatment method for integrated pest management. Longer treatment periods (3 min) and higher voltages (70kV) had a substantial effect on the mortality and fertility rate of cowpea weevils ($P<0.05$). Fertility was lowered in mature adult female cowpea weevils and mortality increased at all three stages of life. Plasma process management is a modified atmospheric gas (65% O_2, 30% N_2, and 5% CO_2), that can generate adequate toxicity to handle the range of insect lifecycle stages, that are disease vectors and pose difficulties for grain integrity in storage. The capacity to employ this technology to reduce storage pests and increase grain quality has the potential to revolutionize the agricultural industry.

DEDICATION

My thesis is dedicated to my family and many friends. A heartfelt thanks to my loving parents. My mom, Jerrell Kirk, and Dad, Timothy Bradley, whose words of support and drive for perseverance continue to resonate throughout my life. Throughout this research project, my friends Amina, Brooke, Tyler, and Will, among others, have been solid foundations for me to depend on. They've been there for me, from late-night practice presentations to revising and brainstorming research techniques. They've been tremendously supportive.

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Contributors

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NOMENCLATURE

ACP	Atmospheric cold plasma
DBD	Dielectric barrier discharge
RGS	Reactive gas species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RCS	Reactive carbon species
Cowpea Weevils	*Callosobruchus maculatus*
IMR	Instantaneous mortality rate
DMR	Delayed mortality rate
IPM	Integrated pest management
DEFRE	UK Department for Environment
FAO	Food and Agriculture Organization
UNEP	United Nations Environment Program
OP	Organophosphate
IGR	Insect growth regulator
LD -50	Lethal dose
DPIRG	Department of Primary Industries and Regional Development
SDState	South Dakota State University Extension
IAEA	International Atomic Energy Agency

KEYWORDS

Pesticides, Environmental impacts, Ecology, IPM, Reduction of pesticides, Crop protection, Atmospheric Cold Plasma, Storage Pest, Mortality, Insecticidal Effect, Dielectric Barrier, Integrated Pest Management, and Reactive Gas Species Hypoxia, Electricity, Reactive Gas Species, ACP Mechanism, *Callosobruchus maculatus*, Anaerobic metabolism, Behavior, Morphology

HIGHLIGHTS

1. At all life cycles, Cowpea weevils were found to have a significant mortality rate when exposed to reactive gas species.
2. Longer treatment times (3 min) and higher voltages (70 kV) reduced cowpea weevil mortality and fertility ($P < 0.05$).
3. Reactive gas species slow the progression of key life cycle phases in cowpea weevils
4. Insects have a remarkable range of adaptations that allow them to cope with varying degrees of hypoxia.
5. After 24 hours, treated cowpea weevils died at a rate of less than 8%, compared to 0% for controls in the hypoxia test. Cowpea weevils at all life cycles were found to have a significant mortality rate when exposed to reactive gas species and electricity.
6. ACP treatment inhibits the fertility rate of adult cowpea weevils by ≥ 50%.

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CHAPTER I

GRANT PROPOSAL: RESEARCH HYPOTHESIS AND OBJECTIVES

Problem Identification, Importance, and Preservation of Agriculture and the Environment

Atmospheric cold plasma (ACP) is an innovative method for extending a product's shelf life by removing microbiological threats (Selcuk et al., 2008; Schmidt et al., 2019. This study assesses the most recent research on cold plasma, with a focus on its possible application in the control of postharvest grain storage pests. Cold plasma is an ionized gas that contains reactive oxygen and nitrogen species, electrons, and free radicals, all of which are toxic to microorganisms (Selcuk et al., 2008; Schmidt et al., 2019). As a safer alternative to conventional pesticides, it has the potential to successfully disinfest stored grains.

The purpose of this study is to determine the efficacy and critical parameters of atmospheric cold plasma (ACP) treatment on the storage pest Callosobruchus maculatus (Cowpea Weevil). ACP has been identified as a novel approach for impacting stored grain pests, microbial and insects (Los et al., 2018; McClurkin-Moore et al., 2017; Schmidt et al., 2019; Shi et al., 2017; Ratish Ramanan et al., 2018). However, the impact of ACP treatment technology has only been evaluated on a lab scale and there is still limited knowledge on how commonly stored grain insects are impacted by ACP treatments. Literature shows that there is a need to develop a better understanding of the use of ACP for controlling insect pests and other microbial contamination so that the technology can be improved for potential application, utilization, and implementation on a larger scale (Kwa, 2001; Konesky & Hill, 2009; Keener et al., 2016; Hojnik et al., 2017). Currently, chemical fumigants are used to clean grain, but they are expensive, detrimental to the environment, and they are not an option for organic seeds and grains. Alternatives to fumigation have been studied and found to be successful, such as ozone (Selcuk et al., 2008; Schmidt et al., 2019). Alternative treatment approaches must be researched in the future.

Hypothesis

Based on what we know about ACP treatment and how effective it is at eradicating microbial issues, we hypothesized that ACP could result in a (≥ 90%) mortality rate of Cowpea weevils at three key life stages. We hypothesized that the mechanism for mortality is the RGS and electrical component that is utilized in the ACP treatment. Lastly, we hypothesized ACP treatment inhibits mature cowpea weevils reproduction.

Objective

To test the hypothesis our overall objective is to examine the use of ACP treatment for the removal of stored-product insect pests in grain. This approach would be analogous to using a fumigant, such as phosphine, to fumigate grain infested with insect pests or before shipment, as required by USDA and other phytosanitary requirements. Plasma treatment has been shown in the literature to be effective against stored grain insect pests. However, no research has been done on how plasma treatment may be integrated into grain management to efficiently disinfest stored grain. The precise aims of this proposed effort are as follows, based on the underlying body of knowledge obtained from the literature and the investigators' (PI, co-PI, and graduate student) experiences with ACP applications on reducing microbial loads on porous surfaces.

1. Quantify the efficacy of ACP treatment on three critical life stages (egg, first instar, and adult) of cowpea weevils.
2. Determine the ACP system's critical component for insect mortality [gas species, hypoxia, or electrical voltage].

3. Identify if ACP treatment inhibits the reproduction of mature cowpea weevils

This proposal addresses NC-213 objectives 2 and 3 by (1) developing methods to maintain quality, capture value, and preserve food safety at key points in the harvest to end product value chain and (2) quantifying and disseminating the impact of market-chain technologies on providing high value, food-safe, and bio-secure grains for global markets and bioprocess industries.

The successful completion of this research will allow us to proceed to the next phase, which will involve collaborating with the agricultural industry to build a test rig for field testing of this concept.

Anticipated Results, Products, and Impacts

The findings of this study will help us establish a reliable treatment technique for using ACP to efficiently disinfect grain against insect pests. If effective, this will have a tremendous influence on the grain sector by giving a viable alternative to the existing limited options for disinfecting grains (Phosphine, EcoFume). This would be especially advantageous to the expanding organic grain business, which is currently constrained by a lack of cost-effective insect pest fumigation options.

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CHAPTER II

LITERATURE REVIEW

Integrated Pest Management Techniques

The integrated pest management (IPM) method includes preventative and corrective strategies to protect pests from generating substantial issues while posing the least danger or harm to humans and desired environmental components (Deguine et al., 2021). Pesticides of various types, (insecticides, herbicides, rodenticides, bactericides, fungicides, and larvicides), have been used for crop protection for years (Deguine et al., 2021). Pesticides help crops, but they also have a significant detrimental influence on the ecosystem. Excessive pesticide usage has the potential to destroy biodiversity; many birds, aquatic species, and animals are threatened by pesticides that are detrimental to their existence (Deguine et al., 2021). Pesticides also pose a threat to the environment's long-term viability and global stability, necessitating the development of alternative pest-control measures. This literature study will discuss common storage pests, insecticides, and pesticide alternatives. This review will also examine how IPM has evolved and evaluate if it is still applicable to present-day challenges.

IPM is regarded as one of the most robust conceptions to emerge in the agricultural industry during the second part of the twentieth century (Deguine et al., 2021). However, IPM has a long history dating back to the late 1800s, when ecology was established as the cornerstone for scientific plant protection (Kogan, 1998). Since the introduction of contemporary organosynthetic pesticides, history has paved the way for a slew of significant scientific and technical advances (Kogan, 1998). IPM has evolved in a variety of ways across numerous nations, and it has expanded outside the realm of entomology. IPM is a good example of how crop protection has progressed over the last six decades. Throughout this time, IPM has worked to promote sustainable agricultural practices, reduce synthetic pesticide usage, and address a variety of socioeconomic, environmental, and human health issues. Pesticide usage throughout the world, on the other hand, has generally remained unabated, with severe consequences for farmer livelihoods, biodiversity protection, and the right to food security (Deguine et al., 2021).

Storage Pest

Pests that feed on stored grain generate significant economic losses and threaten public health by contaminating food (Stejskal et al., 2003). Stored grains are spoiled by a variety of insect pests. Insect and pest infestations are a major cause of food quality changes in foods such as cereal grains and other stored products (Yaseen et al., 2019). Pests attack stored grains more aggressively, resulting in grain weight loss, nutritional value decline, quality deterioration, and seed viability and vigor reduction (Stejskal et al., 2003). Insects are also important vectors for the development of fungal pollutants, which can increase mycotoxin contamination and lower grain quality, making grain quality preservation is a constant concern. Damage to stored grain is easier to categorize by visual inspection of the relative degree of damage and by measuring the amount of weight loss (Stejskal et al., 2003). Grain crop production on a farm is a value-added agricultural operation that encompasses both field planting and grain storage. Many biotic and abiotic elements, as well as their interactions, influence the economic outcome of this process (Stejskal et al., 2003). Arthropod infestation of grain is one of the most economically important biotic variables since these pests cause massive losses of stored goods each harvest across the country (Stejskal et al., 2003). Invasive insects harm the world economy at least $70.0 billion each year, with health expenses exceeding $6.9 billion. Total costs rise as the number of estimates rises; as a result, the real costs of invasive insects to human civilization are much higher (Bradshaw et al., 2016)

It is critical to understand grain storage conditions and how they might encourage pests to reproduce in massive quantities. For example, the cowpea weevil, the subject of this study, has a lifetime of 10 to 14 days (Bean Beetle Handbook, 2005-2021). The pest eats, mates, and dies in its short lifetime. These pests will mate more effectively if the temperature is around 27° C and the relative humidity is around 65% (Bean Beetle Handbook, 2005-2021). Pests that feed on entire kernels in stored products are common. The granary weevil, rice weevil, and Angoumois grain moth are all examples of these pests. Rice weevils are reddish-brown to black snout beetles that are 1/8 to 1/4 inch long. Adults can survive for six to eight months and can be located far from affected products (University Kentucky Entomology). The Angoumois grain moth is a pale yellow-brown grain moth that is 1/2 inch long. The larval stage grows in entire kernels or caked grain, just as the weevils. Infested seeds include barley, rye, corn, oats, rice, and a variety of other grains. The insect is commonly seen in decorative ear corn. It takes roughly 6 weeks for a life cycle to complete (University Kentucky Entomology).

Processed grains or cracked kernels, as well as a range of spices, attract a significantly higher number of insects (Kumar et al., 2017; Neme et al., 2017). Red and Confused flour beetles, Sawtoothed grain beetles, Drugstore beetle, Cigarette beetle, and Indian meal moth are all common pests. Flour beetles, Cigarette beetles, Pharmacy beetles, and Sawtoothed grain beetles are red-brown insects that are 1/8 inch long. The immature or larval stages are only found in contaminated items and are rarely visible. Flour beetle*s* and the Sawtoothed grain beetle cannot consume whole or undamaged grains, but they'll devour dried fruits, dry dog food, dried meats, candy bars, medicines, cigarettes, and several other things (University Kentucky Entomology). The Flour beetles' life cycle lasts around 7 weeks. Females can survive for several months to over a year as adults. Confused flour beetles fly and are drawn to light; red flour beetles crawl toward the light but do not appear to fly. Sawtoothed grain beetles do not fly and are not drawn to light. Several studies on ACP treatment for red flour beetles have been conducted.

Cowpea Weevils

Cowpea (*Vigna unguiculata* (L) Walpers, Fabaceae) is a widely grown edible legume crop in many parts of the world, particularly in tropical and subtropical areas. Due to its high protein content, it is utilized for its nutritional value as well as a livestock feed to create silage and hay. (Diouf, 2011). After maize, *Zea mays* L., and grain sorghum, *Sorghum bicolor* (L.), cowpea is Botswana's third most significant crop Cowpea production in Botswana is at 300–355 kg/ha (Machacha et al., 2012). It's a trifoliate-leaved annual herbaceous legume. It's a drought-tolerant, short-season warm-weather crop that's well-suited to drier climates where other food legumes fail. It needs 750–1100 mm of yearly rainfall (Singh, 2011; Skerm et al., 1988). Insect pests and disease infestations harm cowpea output, resulting in financial losses. Insect damage is the most significant limitation to cowpea grain yield in most producing countries (Singh et al., 1979). Cowpea aphids (*Aphis craccivora*), leafhoppers (*Empoasca spp.*), thrips (*Megalurothrips sjostedti*, flower consuming beetles (*Mylabris spp. and Coryna spp.*), blister beetles (*Hycleu slugens*), green stink bugs (*Nezara viridula*) and cowpea weevil (*Callosobruchus maculatus*).

The cowpea weevil, *Callosobruchus maculatus* (Coleoptera: Bruchidae), is a major pest of legume (Fabaceae) seeds in both the field and storage (Bean Beetle Handbook, 2005-2021). This pest is found all over the world (Bean Beetle Handbook, 2005-2021). The species originated in Africa, according to biogeography. Later, it expanded to tropical and sub-tropical regions of the globe (Beck and Blumer 2014). Crop infestation begins in the field (Beck et al., 2014)), with the majority of damage occurring during storage. On cowpeas, Vigna unguiculata (L.), cowpea weevils is the most destructive, causing yield reductions of up to 90%. (Caswell 1981). Cowpea weevil's populations can increase rapidly, resulting in severe losses in seed weight, germination viability, and crop market value. (Beck et al., 2014). Various approaches and control methods have been established, and more are continually being developed, to prevent major losses encountered during storage (Ziuzina et al., 2021; Schmidt et al., 2019; Brayfield et al., 2018; Wang et al., 2018;). Chemical pesticides are used extensively to control cowpea seed storage pests. However, due to financial and technological constraints, the majority of small-scale farmers have yet to adopt these new practices. Insecticides are also harmful to the environment, people, and non-target species. As a result, there is a need to create low-cost, safe, and simple ways to safeguard stored cowpeas against the cowpea weevil.

During its lifetime, a single female cowpea weevil lays about 100 eggs, one egg per seed. Transparent, oval, or spindle-shaped eggs are adhered to the seed surface (Figure 1). (Beck et al., 2014). At 50X, these are tiny, measuring 0.74 mm in length and 0.38 mm in width. Mitchell (1975) proposed that laying numerous eggs on each seed lengthens the life cycle of the female bean beetle. Adults do not need food or water, and they spend their 10–14-day lifespan mating and laying eggson beans. Adult females will lay single fertilized eggs on the bean's exterior surface once they havebeen inseminated (Beck et al., 2014). Larva that hatches from the egg burrows from the egg through the seed coat and into the bean endosperm without moving outside the protection of the egg (Beck et al., 2014). Once the larva burrows into the bean, the remaining eggshell on top of the cowpea becomes opaque white as it fills with feces from the larva (Beck et al., 2014). The larva burrows and feeds on the bean endosperm and the embryo undergo a series of molts and burrows to a position just underneath the seed coat before pupation (Beck et al., 2014). Although the seed coat of the bean is still intact, around a 1-2mm window is apparent at the location where the beetle is pupating (Beck et al., 2014). There are four larval instars, each of which feeds inside the endosperm of the seed where the egg was deposited (Beck et al., 2014). Adults emerge from the seed by gnawing and extracting a circular piece of the seed coat to create a round exit hole (Beck et al., 2014). This mechanism is damaging to mature cowpeas because it eventually destroys the seed from within.

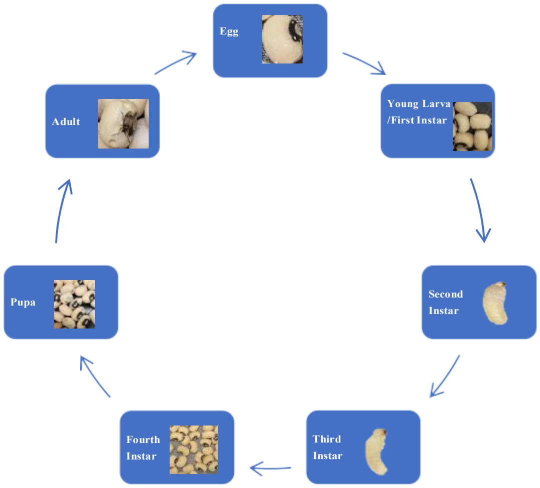

Figure 1. Cowpea Weevil Lifecycle Diagram

Positive and Negative Effects of Synthetic Pesticides and Pesticide Alternatives

By deviating from the essential IPM principles, integration of practices has taken haphazard paths, proven ineffective, and resulted in unsatisfactory consequences (Deguine et al., 2021). Researchers show that chemical management remains the foundation of plant health strategies in the vast majority of situations (Deguine et al., 2021). The agriculture industry is critical to national economies as well as the global economy. Agrarian environments are today considered both economic and ecological ecosystems. The agro-ecosystem is so important because it is both a food and an energy source for the new economy (Magasumovna, 2017). As a result, the desire for increasing output has risen due to the industry’s necessity. William Gaud was the first to implement the widespread use of synthetic insecticides in 1968, during the Green Revolution (Jain, 2010) and since then the heavy use of synthetic pesticides has been normalized. Pesticides have created significant problems, according to researchers such as Dr. Conway. Human morbidity and mortality are rising, while pest populations are increasingly resistant and eluding natural control (Conway, 1997; de Schutter et al., 2011). However, production, performance, and profitability continue to be the top priorities (Horlings et al., 2011).

The world's total land area of 150 million km^2 is divided into 10% arable land, 55% meadows, pastures, and woods, and the rest is unsuitable for agricultural use (Ehrlich, 1968; Brown, 1998). Most of that arable output is based on "conventional" agricultural practices (i.e., production practices that include synthetic agrochemical additives) that have continued to fulfill the demands of human population expansion. Globally, the population has doubled since 1960 (UNEP, 1960) and agricultural output has increased by 2.6 times, yet arable land under cultivation has expanded by just 10% (FAO, 2004a). The implications of agricultural intensification are becoming increasingly apparent. Intensive agriculture is the primary anthropogenic source of ammonia, which is the primary cause of acid rain. It also causes air and groundwater pollution, eutrophication of water systems, and greenhouse gas emissions. Agriculture's scope and tactics (though not necessarily pesticides) have resulted in widespread and persistent biodiversity loss in many areas (Heaney et al., Krebs et al., 1999; Anon., 1999a; Davidson et al., 2001, 2002).

Agriculture functions in an increasingly free-market economy across the world, yet it is still extensively subsidized in several big wealthy countries. Weather, demand, supply, and competition all play a role, and profit takes precedence above social necessity. Over 2.3 billion people (or 30%

of the world's population) lacked year-round access to sufficient food (WHO, 2021). By 2050, this system, with all of its associated environmental challenges, will have to feed a global population of more than 9 billion people. As many northern hemisphere countries opt out of agricultural self-sufficiency (or are forced to), considerable output shifts to the developing globe. The following fight to sustain agricultural earnings in both the developing and developed worlds intensifies and promotes the "tragedy of the commons," a conflict between farmers and the environment (Hardin, 1968).

With the rising globalization of food production comes a communal obligation to maintain farming communities' lives and earnings while protecting current biodiversity and farming landscape's "ecosystem goods and services." The ecological repercussions of pesticide usage are a serious concern in the environment. Insecticides are most commonly linked with environmental harm, even though other parts of contemporary agriculture typically have a bigger environmental impact. Because their stated goal is to kill pests, they may have deadly or sublethal effects on non-target creatures (such as organisms that recycle soil nutrients, pollinate crops, and feed on pest species), as well as diminish and/or pollute food sources for organisms at higher trophic levels.

Current Pesticides Usage

In Uganda, optimal cowpea (*Vigna unguiculata* L. Walp) yields are impeded by a slew of insect pests that attack and harm the crop both in the field and during storage (Machacha et al., 2012). Insecticide treatment is one management strategy that can significantly minimize crop losses caused by insect pest infestations. Synthetic pesticides can effectively reduce insect pest infestations on cowpea. Synthetic pesticides, on the other hand, are costly and may have negative environmental consequences. Natural pesticides are insecticides that are generated entirely from natural sources with little to no chemical modification. Synthetic pesticides are chemicals that have been altered in some way (Matlock et al., 2002). Synthetic insecticides are divided into several categories. Organochlorines, organophosphates, carbamates, and pyrethroids are the primary classes (US EPA).

Pesticide products contain both "active" and "inert" ingredients. An "active ingredient" is a plant regulator, defoliant, desiccant, or nitrogen stabilizer that prevents, eliminates, repels, or mitigates a pest (US EPA). By federal law, all additional substances are referred to as "inert ingredients." They are critical to the performance and usefulness of a product (US EPA). The compounds in a pesticide product that operate to control pests are known as active ingredients. On the label of a pesticide product, active chemicals must be listed by name as well as their proportion by weight (US EPA). Active compounds are classified into various categories Conventional pesticides are those that do not contain biological or antimicrobial pesticides (US EPA). Antimicrobials are compounds or mixes of substances used to eliminate or restrict the growth of hazardous microorganisms on inanimate objects and surfaces, such as bacteria, viruses, or fungus (US EPA). Biopesticides are a type of substance obtained from natural sources (US EPA). Pesticides include at least one active chemical as well as several inert substances that have been purposefully added. The federal government refers to them as "inert ingredients," and they are mixed with active components to create a pesticide product. Chemicals, compounds, and other substances, including common food items and certain natural materials, are examples of (US EPA).

Phosphine gas has been utilized as an effective fumigant for disinfesting stored grains and other commodities for more than four decades around the world. With recent constraints on the manufacturing of the only alternative, Methyl bromide, its usage as a safe fumigant of stored items has become even more crucial (US EPA). Phosphine is a good penetration agent, although it kills insects slowly (at least three days) by interfering with their respiration. The spiracles of an insect do not need to be open, but oxygen is required for Phosphine (PH_3) to work. High doses can result in narcosis and lower insect mortality (Berners et al., 2005). Similar to Phosphine(PH_3), Methyl bromide (CH_3Br) is an effective treatment because it can penetrate the exoskeleton of the insect, cause paralysis, and result in insect death. As effective as these two common pesticides are, Methyl bromide (CH_3Br) and Phosphine (PH_3) fumigants are damaging to the environment (T. Moiseev et al., 2014; M. Ferreira et al., 2016). Methyl bromide (CH_3Br) added to the atmosphere by humans contributes to the thinning of the ozone layer, allowing increased UV radiation to reach the earth's surface (US EPA). Phosphine (PH_3) is a respiratory poison. It affects the transport of oxygen and interferes with the utilization of oxygen by various cells in the body if inhaled (US EPA).

According to the Animal and Plant Health Inspection Service (APHIS), a division of the United States Department of Agriculture (USDA), the United States spends about $120 billion each year in damages of crop production due to the infestation of pests (2015). According to Pimentel (2005), pesticide usage in US agricultural systems yields around $4 for every $1 spent on pest management. As beneficial as this is, the environmental costs of pesticides are not comparable. In the United States, the annual environmental and social costs of agricultural pesticide usage total $10 billion, with $2 billion spent on water surveillance and chemical cleanup alone (Pimentel, 2005). Crop and animal revenues in the United States are over $200 billion per year, accounting for around 4% of total agricultural revenues (Fare et al., 2006).

Pesticide exposure kills 20,000 individuals every year, according to the World Health Organization (WHO) (WHO, 1990), yet the chemicals also safeguard harvests, earnings, and public health. In certain systems, insecticides have been demonstrated to destroy natural enemy populations

(Matlock et al., 2002), while there appear to be limited consequences in others, particularly with some of the newer insecticides (Naranjo et al., 2004). Insecticides have had significant effects on predatory bird populations in certain cases (Sibly et al., 2000), but others have been used in ecologically sensitive habitats for decades with no indication of non-target consequences (Resh et al., 2004; Holmes, 1998). Some have been used so extensively that resistance has hampered their usage within generations (Ishaaya et al., 1995; Devine et al., 2001; Zhao et al., 2002), whereas resistance to others is rare or easy to control.

Despite growing knowledge of the risks associated with their usage, the area treated with pesticides in affluent countries has remained constant over the previous decade. Between 1990 and 2003, roughly 6,000,000 hectares of arable land in the United Kingdom were treated (Anon., 2003b). This is the equivalent of a fourth of the country's overall landmass. Multiple applications to the same places are reflected in the data. Between 1992 and 2001, California treated 6–8,000,000 hectares every year (Epstein et al., 2003; Wilhoit, 2002; et al., 1999). Because newer compounds tend to have better inherent insecticidal action, the overall weight of active chemicals employed has decreased. The LD50 of the organophosphate profenofos against high-risk populations of the silverleaf whitefly (*Bemisia tabaci*) is around 4 ppm, but the LD50 of the juvenile hormone analog (JHA) pyriproxyfen against the same *Bemisia tabaci* strain is 1000 times lower (El Kady et al., 2003). In toxicology, the median lethal dose, LD_{50} is a measure of the lethal dose of a toxin, radiation, or pathogen.

Pesticide usage is not on the decline, even in industrialized nations with strong environmental regulations and aggressive advocacy groups. Synthetic pesticides, especially for high-value crops like vegetables, remain a primary line of protection in most pest control systems, integrated or not.

Alternative Methods

Current synthetic pesticides have drawbacks such as long exposure times and ineffectiveness against a wide range of stored grain pests. Furthermore, the ongoing use of non-target toxicity pesticides is detrimental to soil, terrestrial and aquatic ecosystems, humans, and other living things. They also contribute significantly to pest resistance. Pyrethroids, for example, are very harmful to fish and invertebrates such as lobsters, shrimp, oysters, and aquatic insects. A pyrethroid is an organic substance that is analogous to natural pyrethrins, which are formed by the blooms of pyrethrums. Pyrethroids are commercial and domestic insecticides. Synthetic pyrethroids, which were first launched in the late 1970s (Elliot et al., 1978), currently account for 20% of worldwide pesticide sales and have greatly improved mammal and bird toxicity profiles. Over the last 15 years, novel pesticide classes have been produced, including some that have particularly specific action against certain arthropod orders.

In the industrialized world, these newer chemicals, which the Environmental Protection Agency refers to as "reduced-risk" insecticides, are being used in an increasing percentage of treatments (EPA). Although total agricultural pesticide usage in the United States did not decrease between 1992 and 2000, the use of the "riskiest pesticides" decreased by 14% by weight of active ingredient, according to a Government Accounting Office analysis (GAO, 2001). Insecticide usage patterns are evolving in other emerging countries as well, although the adjustments are slow. Nevertheless, few pesticides are completely safe for the environment. Recently discovered substances may potentially prove to be more harmful than the eco-toxicological data packages given during the registration procedure. The EPA refused to allow the pesticide chlorfenapyr to be used in cotton pest management in 2000, during the final phases of registration, due to the long-term risk it posed to bird reproduction (EPA, 2006).

Natural plant-derived components, entomopathogenic, insect growth regulators, and atmospheric cold (ACP) are a few alternative methods to regulate storage pest infestations. Conventional pesticides are essential for successful pest control, but they can leave residues in grains and promote the spread of resistant stored pest populations. Natural alternatives are preferred since they do not have the same or less harmful side effects as synthetic pesticides. Diatomaceous earth (DE) is a naturally occurring siliceous sedimentary mineral compound derived from the tiny skeletal remnants of diatoms, which are unicellular algae-like plants. Atmospheric cold plasma (ACP) is a nonthermal food processing technique that uses energetic, reactive gases to kill pathogens on meats, poultry, fruits, and vegetables. Recently, ACP treatment has proven to be an effective method for controlling storage pests.

Atmospheric Cold Plasma

Atmospheric Cold Plasma (ACP) is produced by the application of electrical energy to a dielectric barrier discharge (DBD) device. Ions, electrons, neutral species, and UV-visible light make up plasma, which is an ionized gas. ACP systems are made up of two metal electrodes, dielectric barriers, and a transformer that provides electric energy. (Figure 2). When ionizing gas in an ACP system, over 75 unique chemical species, and 500 chemical reactions occur at four different time scales (nano-, micro-, milli-, and seconds) (Misra et al., 2018). For air, this process generates ozone, nitrogen oxides, peroxides, and other reactive gas species (RGS) (Misra et al., 2018).

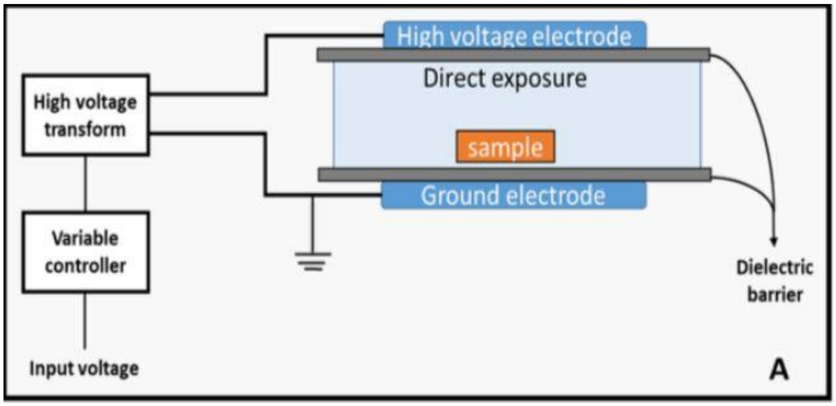

Figure 2. Schematic of experimental lab setup using dielectric barrier discharge atmospheric cold plasma system "Reprinted from [Moore, 2015]."

The major advantage of ACP is its ability to provide a non-thermal treatment that can reduce the presence of chemical residues, pesticides, and environmental pollutants commonly found in cereal crops (Selcuk et al., 2008; Schmidt et al., 2019). Treatment with an ACP system can serve as a bio-decontamination and sterilization process for food, water, living tissues, and even medical equipment (Schmidt et al., 2019). The extension has minimal impact on quality attributes such as color, texture, and sensory, making this technology an ideal solution for many agricultural commodities. For packaged treatments, ACP can be induced in ambient conditions with the input of energy causing the neutral gases inside the packaging material to ionize (Brayfield et al., 2018).

Limitations to Atmospheric Cold Plasma

ACP treatment is still a relatively new technique being researched to aid the agriculture sector. There is not a lot of previous data or analysis to build on and use as a blueprint. According to one study, there were no changes in the levels of SOD and CAT activity for treated insects (Ziuzina et al., 2021). Superoxide dismutase (SOD) and catalase (CAT), two key antioxidant enzymes found in insects, are linked to their natural eating patterns and sensitivity to prooxidant plant allelochemicals, quercetin (a flavonoid), and xanthotoxin (a photoactive furanocoumarin) (Ziuzina et al., 2021). That is one area that needs to be improved. More study is required to investigate long-term plasma effects as well as plant microbiome insect interaction reactions, taking into consideration insect target adaptation and resistance mechanisms, to harness this technology for sustainable biocontrol in the agricultural sector. Furthermore, while studies have concentrated on *Tribolium castaneum* as the target storage pest, a wide variety of storage pests contribute to the degradation of stored grains. This is an area where other researchers can contribute to the efforts of disinfecting stored grains with ACP. *The integration of ACP within the An Alternative Treatment Technology to be Integrated into Pest Management Processes*

Insects are key vectors for the spread of fungal pollutants, which can increase mycotoxin contamination and reduce the quality of stored grains making grain quality preservation an ongoing issue. Pesticides currently on the market have drawbacks, such as long exposure times and ineffectiveness against various stored grain pests.

Multiple studies investigated alternative methods to use chemical pesticides (Ziuzina et al., 2021; Schmidt et al., 2019; Brayfield et al., 2018; Wang et al., 2018;). The use of ACP harnesses a variety of mechanisms and is now being researched further. However, as an insecticidal approach, it is still relatively new, underexplored, and underappreciated. The goal of this study is to see how effective ACP is against cowpea weevils. This storage pest consumes a range of caloric foods consumed by people and is a persistent pest that has a significant impact on grain. This study will look at the effectiveness of ACP on cowpea weevils at three different phases of their lives: egg, first instar, and adult. Furthermore, this research will establish that the RGS is a vital component in the death of insects and that ACP reduces the rate of insect reproduction.

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CHAPTER III

OBJECTIVE 1: EXAMINE THE EFFICACY OF HIGH-VOLTAGE ATMOSPHERIC COLDPLASMA TREATMENT (ACP) FOR THE REMOVAL OF STORED-PRODUCT INSECT PESTS IN GRAIN.

Abstract

Atmospheric cold plasma (ACP) has proven to be a novel technique for prolonging the shelf-life of feed and food products, with potential implications on stored pest insects. Cold plasma ionizes gas thereby producing reactive oxygen and nitrogen species, electrons, and free radicals that destroy and degrade organisms. *Callosobruchus maculatus* (cowpea weevil), is a common pest in stored grains. This pest attacks stored grains, causing grain weight loss, nutritional value decline, quality deterioration, and seed viability and vigor to decline. As a safer alternative to conventional pesticides, ACP has the potential to successfully disinfect stored grains. This research investigated ACP treatments (20, 50, and 70 kV at 1, 2, and 3 minutes) as a viable insecticidal alternative for stored cowpea eggs, first instar larvae, and adults. The ACP treatments resulted in mortality rates at 3 minutes between 50-70 kV of 82% - 93%, 59% - 99%, and 100% mortality by day 6. The ability to use this technology to control storage pests and improve grain quality has the potential to transform the agricultural industry.

Introduction

Baskets, jars, and sacks gave way to more advanced grain storage bins as small-scale storage evolved. These bins protect grain from the elements by elevating it above the ground. Grain bin efficiency has been improved further over time, with features such as perforated floors for retrofitting existing crop storage to newer models, humidity and temperature regulation mechanisms, and varying bin depths (S. K. Pankaj et al., 2013). While this evolved storage practice benefits today's agricultural experts and farmers, the grain they store remains the same, as does the risk of pest infestation. An estimated 80% of the human diet is made up of numerous grains and beans (D. Richard-Molard et al., 2003). Such foods include cereal grains, wheat, maize, rice, barley, and sorghum (D. Richard-Molard et al., 2003). These foods account for a significant portion of human caloric intake. Plant diseases and pests, on the other hand, contribute to 40% of global agricultural produce losses (D. Richard-Molard et al., 2003). Pests attack stored grains more aggressively, resulting in grain weight loss, nutritional value decline, quality deterioration, and seed viability and vigor reduction (M. F. A. El-Aziz et al., 2014). Insects are also important vectors for the development of fungal pollutants, which can increase mycotoxin contamination and lower grain quality, making grain quality preservation and food availability a constant concern (M. F. A. El-Aziz et al., 2014).

Cowpea Weevil

Figure 3. Mature male adult cowpea weevil

Callosobruchus maculatus, also known as the cowpea weevil, is a tropical and subtropical agricultural pest native to Africa and Asia (Beck et al., 2014) . The larvae of this species only eat and grow on the seeds of legumes (Beck et al., 2014). Adults do not need food or water, and they spend their 10-14 day lifespan mating and laying eggs on beans (Beck et al., 2014) . Adult females will lay single fertilized eggs on the bean's exterior surface once they have been inseminated (Beck et al., 2014). Larva than hatches from the egg burrows from the egg through the seed coat and into the bean endosperm without moving outside the protection of the egg (Beck et al., 2014). Once the larva burrows into the bean, the remaining eggshell on top of the cowpea becomes opaque white as it fills with feces from the larva (Beck et al., 2014). The larva burrows and feeds on the bean endosperm and the embryo undergo a series of molts and burrows to a position just underneath the seed coat prior to pupation (Beck et al., 2014). Although the seed coat of the bean is still intact, around 1-2mm window is apparent at the location where the beetle is pupating (Beck et al., 2014). There are four larval instars, each of which feeds inside the endosperm of the seed where the egg was deposited (Beck et al., 2014). Adults emerge from the seed by gnawing and extracting a circular piece of the seed coat to create a round exit hole (Beck et al., 2014). This mechanism is damaging to mature cowpeas because it eventually destroys the seed from within. Cowpeas are high in carbohydrates and proteins, and they also make protease inhibitors to protect themselves from herbivores. Cowpea weevils, on the other hand, develop a slew of digestive enzymes and employ a variety of tactics to get through the plant's defenses.

Current methods for insect treatment (pesticides)

Cowpea Weevils has been controlled by organophosphates, pyrethroids, Pyrethrin’s, phosphine, and other fumigants (T. Moiseev et al., 2014). Pyrethroids are very toxic to fish and invertebrates alike. Pyrethroids are also toxic to lobsters, shrimp, oysters, and aquatic insects (T. Moiseev et al., 2014). Long-term pyrethroid exposure has been proven to affect fish and aquatic insects' reproduction (T. Moiseev et al., 2014). Pyrethrins degrade rapidly in the environment, particularly when exposed to sunlight (M. Ferreira et al., 2016). Pyrethrin’s also irritated your skin if exposed and can induce numbness or tingling at the point of contact. By-products of organophosphates pollute soil, causing fertility loss, acidification, nitrate leaching, and weed resistance. In the environment, phosphine degrades quickly. These are just a fraction of the side effects of existing fumigation techniques for storage pest management.

The insect studied in this paper, cowpea weevil, has evolved resistance to a variety of insecticides, including phosphine, in numerous countries (M. Ferreira et al., 2016). As a result, new strategies are required to assist in successfully disinfecting stored grains. Ideally, pesticides should only be toxic to target organisms, eco-friendly, and be biodegradable. However, it has been estimated that only 0.1% of the pesticides reach the target organisms and the remaining by-products are integrated into the environment (Carriger et.al, 2006). Repeated use of persistent non-biodegradable pesticides has polluted various components of water, air, and soil ecosystems. Pesticides have also entered the food chain and have bioaccumulated in the higher tropic level.

Current pesticides have limitations such as extensive exposure times and ineffectiveness against a variety of stored grain pests (M. F. A. El-Aziz et al., 2014). In addition, continued use of non-target toxicity pesticides has significant consequences on soil, terrestrial and aquatic ecosystems, humans, and other living things, as well as contributing to insect resistance (Mahmood et al., 2016). Several studies looked into alternatives to pesticides (Huang et al., 2005, Kedia et al., 2015, Souto et al., 2021). Alternative approaches include natural plant-derived components, insect growth regulators, ozone (McDonough, 2010), and (ACP).

Atmospheric cold plasma treatment for stored grain management

ACP is a nonthermal treatment technology that uses energetic, reactive gases to kill pathogens on meats, poultry, fruits, and vegetables (T. Moiseev et al., 2014). This versatile sanitizing approach employs electricity and a carrier gas such as air, oxygen, nitrogen, or helium (T. Moiseev et al., 2014). No antibacterial chemical compounds are required. ACP uses a variety of processes to combat biocontamination and sustainability challenges in the food and agriculture industries. UV photons, balanced, negative, and positive ions, free radicals, and free electrons make up ACP, which is produced at atmospheric pressure and has low or no temperature effects on target foods

(M. Ferreira et al., 2016). These qualities provide a rich resource for developing alternative agricultural commodity preservation solutions to existing fumigation procedures.

ACP is produced by the application of electrical energy to a dielectric barrier discharge (DBD) device. Ions, electrons, neutral species, and UV-visible light make up plasma, which is an ionized gas. ACP systems are made up of two metal electrodes, dielectric barriers, and a transformer that provides electric energy. When ionizing gas in an ACP system, over 75 unique chemical species and 500 chemical reactions occur at four different time scales (nano-, micro-, milli-, and seconds) (Misra et al., 2019b). For air, this process generates ozone, nitrogen oxides, peroxides, and other reactive gas species (RGS) (Gordillo-Vazquez, 2008; Misra et al., 2016).

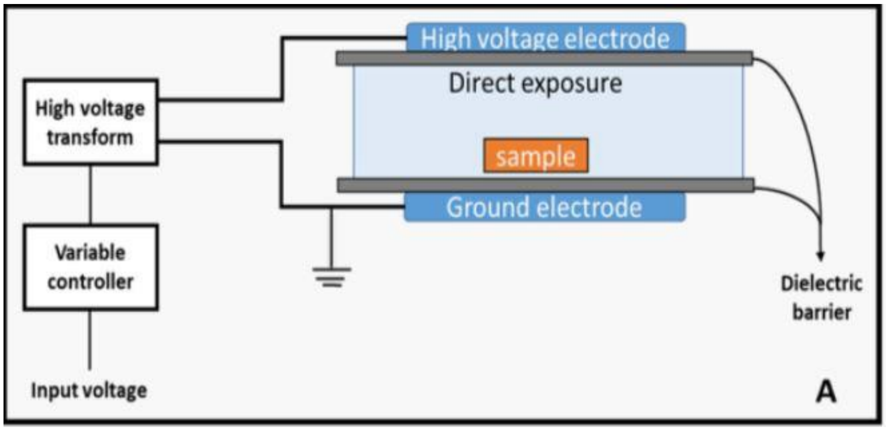


Figure 4. Schematic of experimental lab setup using dielectric barrier discharge atmospheric cold plasma system "Reprinted from [Moore, 2015]."

The major advantage of ACP is its ability to provide a non-thermal treatment that can reduce the presence of chemical residues, pesticides, and environmental pollutants commonly found in cereal crops (Selcuk et al., 2008; Schmidt et al., 2019). Treatment with an ACP system can serve as a bio-decontamination and sterilization process for food, water, living tissues, and even medical equipment. The extension has minimal impact on quality attributes such as color, texture, and sensory, making this technology an ideal solution for many agricultural commodities. For packaged treatments, ACP can be induced in ambient conditions with the input of energy causing the neutral gases inside the packaging material to ionize (Brayfield et al., 2018).

The dielectric barrier discharges ionize the present neutral gases by applying high voltage in an Interdialytic space (Misra et al., 2015). Reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen molecules, superoxide anions, and ozone are responsible for the deactivation of microbes (Hashizume et al., 2013). The reaction mechanisms involve the vibration, excitation, dissociation, attachment, and ionization of the molecular species by causing strong responses to the applied magnetic field (Fridman et al., 2008). Optical emission spectroscopy is used to characterize the ROS and RGS species created during ionization. When attempting to model reactions, ACP treatment is heavily affected by the physicochemical and physiological parameters which include moisture content, protein concentration, nitrogen levels, growth, and overall yield which creates antimicrobial species that react with any microbes that may be formed on the infected commodity that results in the decontamination for a few hours post-treatment (Surowsky, et al., 2013).

While the use of ACP to treat stored grains has yielded positive results (**Table 1**), little study has been done on how the ROS and RGS produced by ACP treatment affect common stored grain insects. This work is required before a full scale up to industrial use of stored commodities. There have been little to no studies on the ability of ACP treatments to diminish cowpea weevils in grain across all lifecycles. Similar to how knowledge advanced with the impacts of ozone as a fumigant alternative, there is still much work to be done on the impact of ACP on common stored grain insects.

Table 1. Past Research on Atmospheric Cold Plasma Treatment of Various Stored Grains and Feed.

Stored Product	Voltage	Freq	Gas Comp	Organism	Log Reduction	Treatment Time	Author
Distillers Wet Grains	70 kV	60 Hz	O2/CO2/N2. Package DBD	Total mesophilic	2.23	6 min	McClurkin Moore., 2017
Wheat Grain	80 kV	50 Hz	Air; Package DBD	Yeast	2.5	20 min	Los et al., 2018
Barley Grain	80 kV	50 Hz	Air; Package DBD	Total mesophilic	2.4	20 min	Los et al., 2018
Maize	10 kV	25 kHz	Air; Plasma Jet	A. flavus and A. parasiticus	5.48 and 5.20	1-5 min	Dasan et al.., 2016
Maize	90 kV	50 Hz	O2/CO2/N2. Package DBD	Aflatoxin	89-90% reduction	30 min	Shi et al., 2017
Brown Rice	10 kV		Argon; Plasma Jet	A. flavus	20-day shelf-life extension	20 min	Suhem et al., 2013b

The overall objective was to examine the efficacy of ACP treatment for the removal of stored-product insect pests in grain. This approach would be analogous to using a fumigant, such as phosphine, to fumigate grain infested with insect pests or before shipment, as required by USDA and other phytosanitary requirements. Plasma treatment has been shown in the literature to be effective against stored grain insect pests (Ziuzina et al., 2021; Donohue et al., 2006; Hassan et al., 2019). However, no research has been done on how plasma treatment may be integrated into grain management to efficiently disinfest stored cowpea seeds.

Material and Methods

The first generation of weevils utilized in this study originated from a colony at Texas A&M University in College Station, Texas, which was maintained in the Entomology Laboratory. The PHEED Laboratory at Texas A&M University in College Station, Texas, was where the remainder of the generations were breaded. The weevils were housed in glass jars covered with porous Kimtech® Wipes and fed 75 grams of cowpea seeds (Vigna unguiculata) daily. The weevils were incubated in an 80.6 ± 1 ° F (27 ± 1° C) room temperature environment with a relative humidity of 60 ± 5% at all phases of their development. Grain for the experiment was purchased from Azure Standard, Dufur, Oregon, a company that produces natural, organic, non-GMO, and environmentally friendly products.

Treatment of Insect Eggs

For these treatments, 125 first-generation mature cowpea weevils (purified colony from Texas A&M University Entomology Lab) were placed in a sealed container with 75 grams of cowpeas and permitted to mate for 1-2 hours.

540posited eggs were taken out of the container with the adult cowpea weevils and placed in a Cryovac ® bag (SEALED AIR®, Charlotte, NC) with an inner width and length of 20 mm x 30 cm, flushed and filled with 4- 5 cm of MAP gas (65% O_2, 30% CO_2, and 5% N_2),heat-sealed and treated. The MAP gas packages containing cowpea seeds with eggs deposited on top of them were exposed to voltages of 20, 50, and 70 kV for 1,2, and 3 minutes in three replications. After treatment, the package sat in a room temperature environment for 30 minutes in the bag with gaseous species and then rehomed until hatched. This study was repeated three times.

Treatment of First Instar Insect Larvae

The larva hatched from the egg after 1-2 weeks and burrowed through the seed coat and subsequently into the bean endosperm without leaving the egg's protection.

Once the cowpea weevils reached the above phase, 540 counted cowpea weevil larvae were placed in a Cryovac ® bag (SEALED AIR®, Charlotte, NC) with an inner width and length of 20 mm x 30 cm, flushed and filled with 4- 5 cm of MAP gas (65% O2, 30% CO2, and 5% N2), heat-sealed and treated. The MAP gas packages containing cowpea seed larvae were exposed to voltages of 20, 50, and 70 kV for 1,2,3 minute in three replications. After treatment, the package sat in a room temperature environment for 30 minutes in the bag with gaseous species and then rehomed and observed until hatched. This study was repeated three times.

Treatment of Adult Insects

216 mature insects were put in a Cryovac ® bag (SEALED AIR®, Charlotte, NC) with an inner width and length of 20 mm x 30 cm, flushed and filled with 4- 5 cm of MAP gas (65% O_2, 30% CO_2, and 5% N_2), heat-sealed and treated. The MAP gas packages containing adult cowpea weevils were exposed to voltages of 20, 50, and 70 kV for 1,2, and 3 minutes in three replications. After treatment, the package was rehomed and examined for a 7-day PTRT period in a room temperature environment in a bag with gaseous species. This study was repeated three times.

Statistical Analysis

SPSS Statistics was used to conduct the statistical analysis (version 27.0.1.0, IBM Software). The means of all ACP-treated samples and their respective untreated controls were analyzed using

SPSS and compared using Fisher's least significant difference at the 0,05-confidence interval. This software also provided the standard deviation, minimum, and maximum values.

Results and Discussion

Analysis of Insects

Percentage mortality was used to assess the impact of ACP treatment on cowpea weevil viability. All of the insects were examined using an Insten Magnifying Glass and the naked eye. To determine the effect of ACP on cowpea weevils, the eggs and first instar larvae were incubated at 27°C with 65% relative humidity in a glass mason jar covered with Kimtech wipes and observed until they hatched and emerged. The hatch rate is the percentage of eggs that made it to the first instar phase after surviving treatment. After around 1-2 weeks from the time the eggs are laid, the first instar phase appears. The egg has entered the first instar phase when the larva burrows into the bean endosperm through the seed coat and a 1-2 mm window develops. To determine whether or not the hatches were successful, the number of windows was recorded. The percentage of eggs (0.75 mm long, oval, translucent, and shiny) that remained securely attached to the bean surface after 2 weeks was reported, showing that eggs died after treatment. The emergence rate was assessed by the number of cowpea eggs that became adults. If the eggs failed to emerge, the percentage of mortality was determined. After roughly a month and a half, when the controls generally emerge, the number of treated eggs that developed into adults was counted. If the first instar larvae did not emerge after 1 month and 12 days, first instar emergence mortality was calculated in the same way as the eggs.

The adults were treated in the same way as the eggs and first instar larvae. Adult insects were examined for IMR and PTRT mortality after treatment. Adults were treated and remained in the

Cryovac ® bag for 30 minutes, flushed, and filled with MAP gas (65% O_2, 30% CO_2, and 5% N_2) before being counted. After the adults were taken from the Cryovac ® bag and placed in a glass mason jar with 1.5 g of cowpea seeds, covered with Kimtech wipes, and examined for 7 days, PTRT mortality was recorded for each day. If the adult insect did not move after being touched with the bristles of a paintbrush, it was declared dead.

The mortality of the eggs was calculated as the number of treated eggs unhatched/ total number of treated eggs hatched per trial × 100%. The mortality of the eggs was calculated as the number of treated eggs not emerged/total number of treated eggs that emerged per trial × 100%. The mortality of the 1st instar was calculated as the number of treated first instar not emerged/total number of treated first instar emerged per trial × 100%. The IMR of the adults was calculated as the number of treated adults who died/total number of treated alive adults per trial × 100%. The PTRT mortality of the adults was calculated as the number of treated adults who died/total number of control adults that died over 7 days per trial × 100%.

The changes in appearance that were analyzed, included size, and physical anatomy. Color changes were observed with the natural eye and documented. The elytra of a typical cowpea weevil are brown with white and black markings. After treatment, some of the cowpea weevils were dark black with barely visible white and black markings on their elytra.

Effect of ACP on the Viability of Cowpea Weevils

The effect of ACP on cowpea weevils was influenced by the amount of voltage, treatment duration, and PTRT. Cowpea weevil mortality increased with increasing voltage and treatment time at all phases of life, as evidenced by other researchers' studies on the insecticidal effects of ACP (Ziuzina et al., 2021; Donohue et al., 2006; Hassan et al., 2019). In this study, the use of direct exposure to plasma was applied. Direct exposure is the combined attack of produced charged particles, UV, and short and long-lived plasma reactive species on target organisms (Ziuzina et al., 2021).

The RGS from the plasma, in general, breaks down cell walls, doubles lipid bonds, and allows ROS to destroy intracellular structures. By causing strong responses to the applied field, the reaction mechanisms involve the vibration, excitation, dissociation, attachment, and ionization of the molecular species. There are studies to support the inactivation of Aspergillus flavus using ACP by showing that there was cell leakage and loss of viability (Suhem, et al., 2013b). This same mechanism, on a cellular level, is damaging to insects. The exposure to the reactive species has shown that post ACP treatment the conidiophores and the vesicles were broken which results in loss of cellular viability, similarly with insects where the double lipid bonds in the cell are disrupted, allowing ROS to react with the inner cellular structures. Cowpea weevils hatch rates were also inhibited by ACP treatment. At 70 kV for 3 minutes the treated eggs averaged a hatch rate mortality of 91%, compared to 10% for controls (Figure 5).

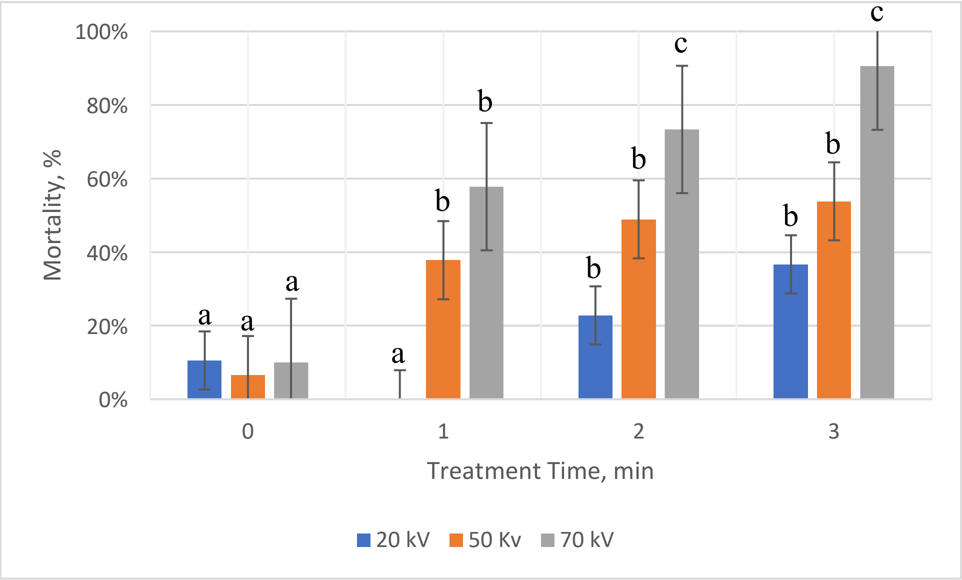
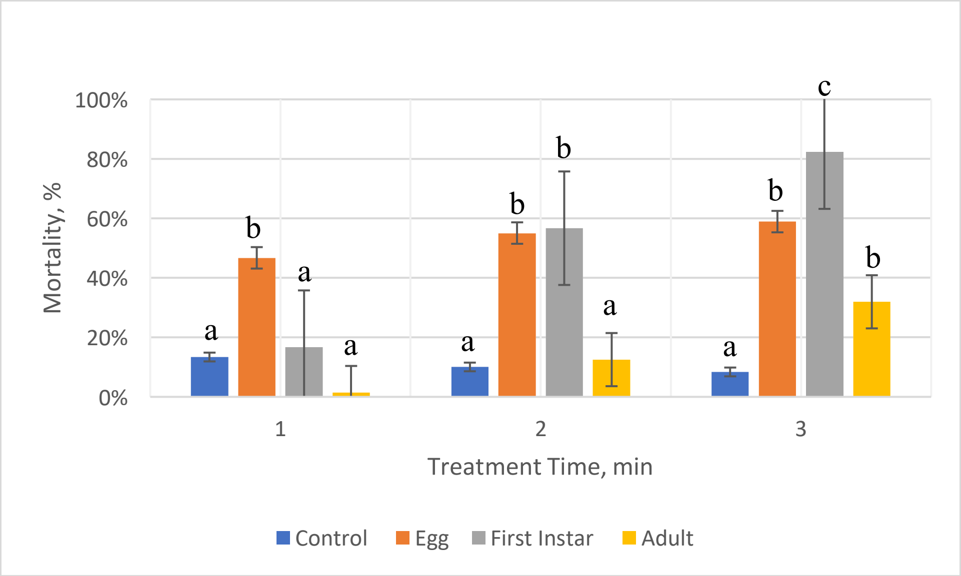


Figure 5. Percent of cowpea weevil's eggs treated with ACP that did not hatch into the first instar. The effects of direct exposure to ACP on cowpea weevil vitality (eggs, first instar larvae, and adults). As a function of treatment time, voltage levels, and post-treatment retention time (PTRT), the mortality of insects was recorded: **(a)** 0 days for cowpea weevil hatch mortality for 0-3 minutes at 20-70 kV, **(b)** EMR at day 0 for 1-3 minutes at 20 kV, **(c)** EMR at day 0 for 1-3 minutes at 50 kV, **(d)** EMR at day 0 for 1-3 minutes at 70 kV., **(e)** PTRT mortality of adults for 1-3 minutes at 20-70 kV (Figures 5- 7). Different letters represent significant differences at the level of $p < 0.05$.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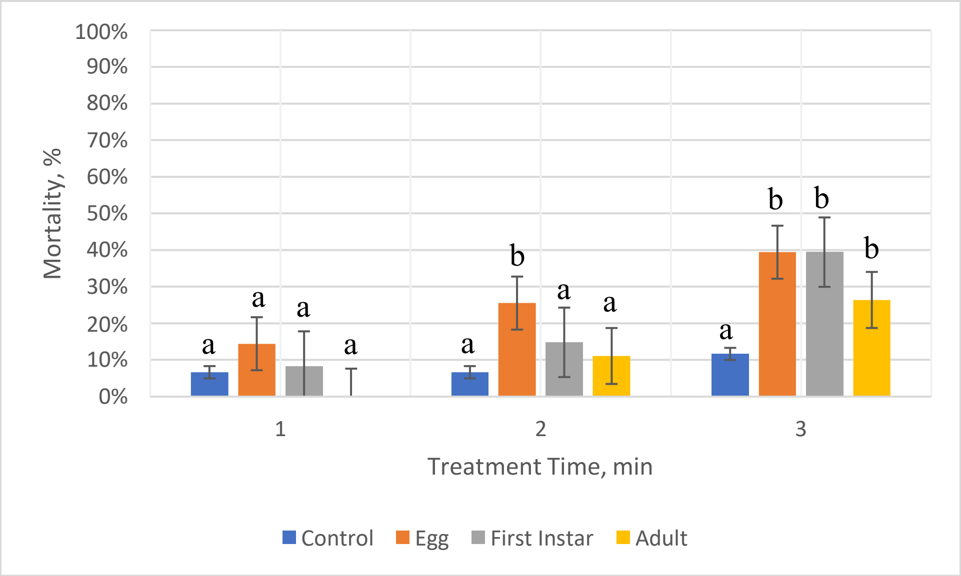
(a)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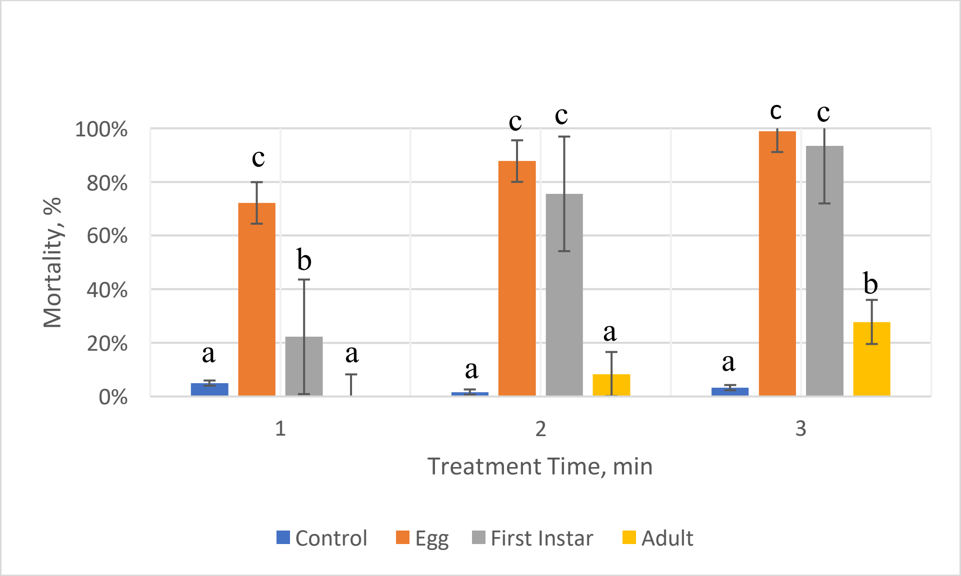

(c)

Figure 6. Percent mortality of cowpea weevils, at three different life stages, treated with (a) 20kV, (b) 50kV, and (c) 70kV for 1, 2, and 3 minutes.

The results for mortality of eggs treated with ACP (**Figure 5**) represent the eggs that hatched to thefirst instar but did not survive to reach the adult stage. (**Figure 6a**) shows that egg, first instar, and adult cowpea weevils treated with ACP had a mortality of up to 39% for 20 kV 3-minute treatmentsof the egg and first instar. The percent mortality increased for the egg and first instar life cycle as treatment time and treatment voltage increased. The most effective treatment for eggs and first instar was 70kV at 3 minutes, with 99% and 93% mortality respectively, compared to 3% for controls. For the initial kill of adults, 50kV at 3 minutes (**Figure 6b**) was the most effective with a 32% mortality rate. At 70kV (**Figure 6c**) the treatment of adults was least effective for an initial kill response.

Adults proved to be more difficult to kill, according to the results of the treatment. Higher treatment times, voltages, and PTRT lengths were found to be effective in killing adult insects. According to a study on the half-life of ozone as a function of air movement and conditions in a sealed container, ozone remained present in the sealed container for 2.4 days after treatment (McClurkin et al., 2013). Longer PTRT was observed, implying that the mechanism of inactivation following ACP treatment should be investigated further. By day 6, direct exposure to ACP for 3 minutes at 70 kV resulted in 100% mortality, compared to 0% mortality in non-treated insects (**Figure 7**). Onday 7, direct exposure to ACP for 3 minutes at 70 kV resulted in 100% mortality, compared to 0%mortality in non-treated insects (**Figure 7**). This is due to the reactive plasma particles, whose effects were amplified by employing a confined plasma treatment method. For egg treatment, shorter treatment times and lower voltages yielded a relatively high mortality rate in comparison to the low treatment times and voltages for the first instar. This difference is because it takes more energy to penetrate the seed's endosperm to get to the first instar larvae.

On day 7, ACP treatment yielded a mortality rate of 100% for all voltages and treatment times. This means ACP treatment reduced the 10–14-day lifespan of the adult cowpea weevil by over half. By day 6, direct exposure to ACP for 3 minutes at 70 kV resulted in 100% mortality, compared to 0% mortality in non-treated insects (**Figure 7**). On day 7, direct exposure to ACP for 3 minutes at 70 kV resulted in 100% mortality, compared to 0% mortality in non-treated insects (**Figure 7**). This is due to the reactive plasma particles, whose effects were amplified by employing a confined plasma treatment method. For egg treatment, shorter treatment times and lower voltages yielded a relatively high mortality rate in comparison to the low treatment times and voltages for the first instar. This difference is because it takes more energy to penetrate the seed's endosperm to get to the first instar larvae. On day 7, ACP treatment yielded a mortality rate of 100% for all voltages and treatment times. This means ACP treatment reduced the 10–14-day lifespan of the adult cowpea weevil by over half.

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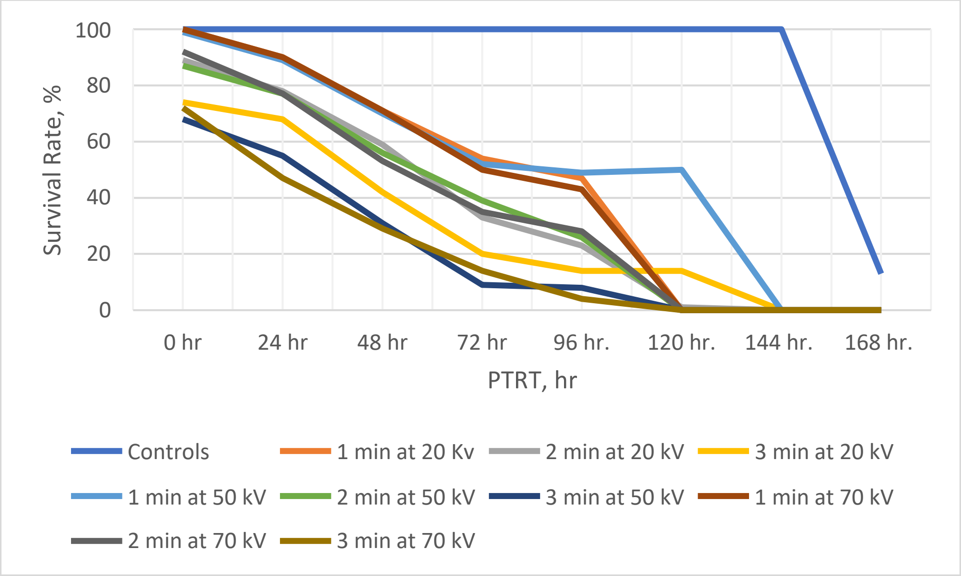

Figure 7. Treated Adults Cowpea Weevils at 20, 50, and 70 kV for 1,2, and 3 min (PTRT - 7 Days)

Direct exposure significantly reduced ($p < 0.05$) the viability of the eggs and larvae at 50-70 kV for 3 minutes. Direct exposure significantly reduced ($p < 0.05$) the viability of adults at 50-70 kV for 3 minutes. The mortalities for the eggs, first instar larvae, and adults were 82% - 93%, 59% -

99%, and 100% mortality by day 6. Cowpea weevils' life expectancy is 10-14 days after emergence.

The findings are directly applicable to the treatment of stored grain commodities, and they are consistent with previous studies (Los et al., 2018; Suhem et al., 2013b; McClurkin Moore., 2017), which showed that the manner of plasma exposure and PTRT in a controlled environment were important factors in attaining biocidal effects. Adults were more susceptible to death when PTRT was applied.

Researchers treated *Tribolium castaneum* at various stages of development to determine the treatment parameters for ACP treatment. Viability assay calculated the percentage of the mortality of the insects at each stage of life (Ziuzina et al., 2021).

Researchers demonstrated that ACP treatment can be used to disinfect stored grains (Ziuzina et al., 2021; Donohue et al., 2006; Hassan et al., 2019). One study demonstrated the efficacy of a confined ACP treatment in the air (Hassan et al., 2019). *Tribolium castaneum* mortality was achieved using relatively short treatment intervals ranging from 0.5 to 5 minutes, with adults being the most resistant stage to direct treatment (Ziuzina et al., 2021). Another study found that adult populations' respiration rates were reduced after being exposed to sublethal ACP treatments and that this was positively related to insect weight (Dana Ziuzina et al., 2021). As a result, as the treatment period grew, so did the levels of lipid peroxidation and the activity of GST.

ACP treatment is still a relatively new technique being researched to aid the agriculture industry. There is limited data and/or analysis to build on and use as a blueprint. According to one study, there were no changes in the levels of SOD and CAT activity for treated insects (Dana Ziuzina et al., 2021). That is one area that needs to be improved. More research is required to investigate long-term plasma effects as well as plant microbiome insect interaction reactions, taking into consideration insect target adaptation and resistance mechanisms, to harness this technology for sustainable biocontrol in the agricultural industry. Furthermore, while studies have concentrated on *Tribolium castaneum* as the target storage pest, a wide variety of storage pests contribute to the degradation of stored grains.

This research shows that different lifecycle stages can impact effective inactivation and emphasizes the necessity for process optimization in which treatments are coordinated with the preservation of other features such as grain functioning. To find viable uses of ACP for integrated pest management methods across the agriculture industry, more research into different stored pests and their distinct lifecycle stages is needed.

Reactive oxygen species (ROS) produced by plasma can trigger the degradation of hydrocarbons in the cuticular lipid layer of insects, as well as dehydration and mortality (Ramanan et al., 2018). The color shift from brown to dark black was evident in sample homogenates (**Figure 7**), even though these changes were not visible in treated adult insects before homogenization. The discoloration might be caused by pigment degradation caused by plasma bleaching. Treatment with ozone can result in considerable changes in fatty acid content as well as the degradation of natural colors (Byun et al., 1997). Dehydration may be one of the variables determining mature cowpea weevil's insect body-color, with hydrated populations being much darker than dehydrated populations (Noh et al., 2015). The body color of the pea aphid was demonstrated to be irrevocably affected by starvation, shifting from red to pale under stress, and this was linked to changes in pigment composition and concentration (Wang et al., 2019). The researchers speculated that the aphids' body-color shift may represent an adaptation mechanism to environmental stress and a technique for storing and using energy reserves. The change in color of the insects' homogenate shows that biochemical changes have occurred as a result of plasma exposure, although it is uncertain whether this is an effective stress response.

Figure 8. Non-treated adult cowpea weevils vs. treated adult cowpea weevils at 70 kV for 3 minutes

The hypothesis in this study was valid since altering the parameters of the ACP system had an effect on the cowpea weevils at all phases of life, resulting in insect death. According to the data, higher voltages, treatment durations, and PTRT for adults had the most impact. However, there is minimal impact at lower voltages and shorter treatment durations. The goal of assessing the efficacy of ACP treatment on three crucial life phases of cowpea weevils (egg, first instar, and adult) was met.

Conclusions

The major advantage of ACP is its ability to provide a non-thermal treatment that can reduce the presence of chemical residues, pesticides, and environmental pollutants commonly found in cereal crops (Selcuk et al., 2008; Schmidt et al., 2019). Treatment with an ACP system can serve as a bio-decontamination and sterilization process for food, water, living tissues, and even medical equipment. The extension has minimal impact on quality attributes such as color, texture, and sensory, making this technology an ideal solution for many agricultural commodities.

This same mechanism, on a cellular level, is damaging to insects.

The exposure to the reactive species has shown that post ACP treatment the conidiophores and the vesicles were broken which results in loss of cellular viability, similarly with insects where the double lipid bonds in the cell are disrupted, allowing ROS to react with the inner cellular structures. We demonstrated ACP can effectively treat cowpea weevils. As a result, this novel treatment technology has the potential to be used and aid in grain storage sustainability. We achieved high mortality rates in three major developmental phases of the cowpea weevil with very short treatment times of 1-3 minutes at voltages of 20, 50, and 70 kV. Although the adult stage was the most resistant to treatment, the predicted mortality rate was nevertheless achieved. To adapt the novel technology for sustainable storage pest management in the agriculture industry, more research is needed to understand insect target adaptation and resistance processes.

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CHAPTER IV

OBJECTIVE 2: ATMOSPHERIC COLD PLASMA (ACP) TREATMENT MECHANISM ON COWPEA WEEVILS

Abstract

Atmospheric Cold Plasma (ACP) is a viable alternative to existing storage pest management processes. The presumed mode of action that causes adult *Callosobruchus maculatus* (cowpea weevil) mortality might be analogous to the lethal impact generated by reactive plasma species in microbial inactivation (Xu, L., Garner, A.L., Tao, B. et al., 2017; Ziuzina et al., 2013; Lu et al., 2014; Xu, L., Yepez, X., Applegate, B. et al., 2020). Plasma degrades the lipids, proteins, and DNA of cells. The ROS species causes oxidation of the lipid bilayer of cellular membranes. Plasma etching causes perforation of cell walls, leading to cell lysis and the charged species plasma causes electroporation of cell membrane, leading to cell leakage and insect death (Xu, L., Garner, A.L., Tao, B. et al., 2017; Ziuzina et al., 2013; Lu et al., 2014; Xu, L., Yepez, X., Applegate, B. et al., 2020). However, it is still unclear if hypoxia, electricity, or reactive gas species contribute the most to ACP treatments and their efficacy in pest mortality. The mechanism of an ACP treatment system was examined in this work against cowpea weevils. Adult cowpea weevils were exposed to three types of treatments. For the hypoxia treatment, groups of 8 mature cowpea weevils in three replicates were heat-sealed in a Cryovac ® bag for 0 – 72 hours at 80.6 ± 1 ° F (27 ± 1° C) room temperature environment with a relative humidity of 60 ± 5% to allow them to metabolize the oxygen present in the bag. After the 0–72-hour periods, the survival rate of the cowpea weevils was recorded. For the atmospheric cold plasma treatments, groups of 8 mature cowpea weevils in three replicates were placed in a Cryovac ® bag and flushed and filled with MAP gas (65% O_2, 30% CO_2, and 5% N_2) and heat-sealed, then treated at 20 – 70 kV for 1-3 minutes. After the 0-72 hour

periods, the survival rate of the cowpea weevils was recorded. For the electrical treatments, 8 mature cowpea weevils in three replicates were placed in a Cryovac ® bag, heat-sealed, and then treated at 20 – 70 kV for 1-3 minutes. After the 0–72-hour periods, the survival rate of the cowpea weevils was recorded. After all the treatments were conducted, the data was analyzed to determine and compare mortality for each method. The most effective treatment was 70 kV of electricity, which resulted in a 92% - 100% mortality rate in 1-3 minutes. The hypoxia treatment, which had a mortality rate of 2% after 72 hours, was less effective. As a result, the RGS and electrical treatments contribute to the ACP treatment system's effectiveness.

Introduction

For thousands of years, cereal grains have been the primary component of human diets, and they have played a significant role in building human civilization. Rice, wheat, and maize, as well as sorghum and millets to a lesser extent, are key staples for billions of people throughout the world. Cereal grain consumption accounts for more than half of the world's daily calorie intake (Gustafson et al., 2009). FAO's latest forecast for world cereal production in 2021 has been lifted by 2.1 million tonnes in February and now stands at 2,793 million tonnes, 0.8 percent higher year-on-year (FAO, 2021). Optimizing the quality and safety of cereal grains and cereal products remains a critical food safety concern due to their global importance and widespread usage as human food and livestock feed. Several factors contribute to microbial contamination and insect damage to wheat grains (Bullerman et al., 2009).

Between a quarter and a third of the world's grain production is lost each year during storage, according to research studies (DPIRG 2019). Insects are to blame for a lot of it. Furthermore, insect damage degrades the quality of grain that is not lost. Many grain pests prefer to consume grain embryos, which lowers the protein content of feed grain and the proportion of seeds that germinate. The smaller grain borer, rice weevil, and rust-red flour beetle are all major pests of stored grains. Consumers from other countries require grain that is free of insects. As a result, the Australian Department of Agriculture has implemented a zero-tolerance policy for insects in export grain. Grain farmers' expenses are also increased by insect pests, both directly on the field and indirectly through the expenditures incurred by grain handling authorities in eliminating weevils in bulk storage (DPIRG 2019). Primary and secondary pests of grain insects can be classified.

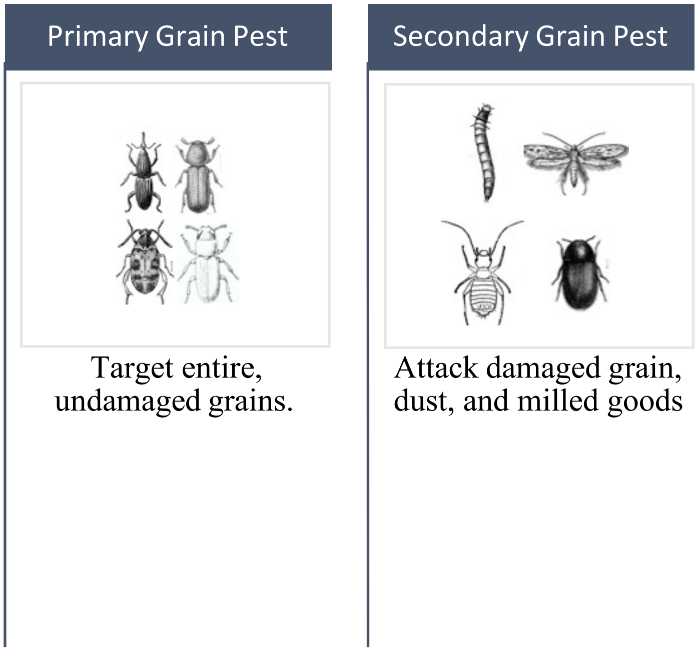


Figure 9. Primary and secondary grain pest comparison

To control these primary and secondary pests, when an insect infestation is detected in stored grain, the grain can be transported and treated with a protectant pesticide, given to animals as-is, sold at a lower market value, or fumigated (SDState, 2020). Considering fumigant insecticides are dangerous, it is strongly advised that they be applied by a trained expert. Many of these products are triggered by moisture, including air moisture. This indicates that the product becomes active the moment the seal is broken, or the package is opened. To avoid any unwanted health effects, including death, fumigants should be applied using forced air or oxygen-supplied respirator system (SDState, 2020). Because of the environmental effects of synthetic pesticides, such as their toxicity to fish and invertebrates, and their byproducts polluting soil, causing fertility loss, acidification, nitrate leaching, and weed resistance (T. Moiseev et al., 2014), there are effective alternatives to pesticides for pest control. Sanitation, monitoring, temperature control, carbon dioxide fumigation, diatomaceous earth, and biological control are among them. Integrating these preventative and intervention measures can help to reduce insect infestations in stored grains. Several studies looked into pesticide alternatives (Huang et al., 2005, Kedia et al., 2015, Souto et al., 2021). Natural plant-derived components, insect growth regulators, ozone, and other alternatives have been proposed (McDonough, 2010; Ziuzina et al., 2021).

Fundamentals of atmospheric cold plasma treatment

The fourth state of matter is known as "plasma." It is a neutral ionized gas made up of ions, free radicals, excited and non-excited atoms, and molecules with a net electrical charge of nearly zero (Bárdos et al., 2010; Pankaj et al., 2014). Crookes (1879) was the first to discover it, and it took over 50 years for Tonks and Langmuir (1929) to effectively create plasma-generating systems. Plasma can be categorized in a variety of ways depending on density, ionization, thermodynamic equilibrium, and other factors. Based on temperature circumstances, plasma can be classified as thermal, non-thermal, or local thermal equilibrium. In the thermal plasma, all species (electrons, ions, and neutral species) are in thermodynamic equilibrium. The temperature of plasma species in non-thermal equilibrium plasmas, on the other hand, is not in the same range.

Among all plasma sources, atmospheric pressure plasma jet (APPJ) and dielectric barrier discharge (DBD) plasmas have the most thoroughly investigated configurations for decontamination and quality studies, and they are simple to adopt and build (**Fig. 9**). They are also more commercially available and simple to employ under atmospheric settings. DBD plasma consists of two electrodes, at least one of which is coated by a dielectric barrier material. By preventing any arc transition from the processing environment, this barrier generates a large number of micro-discharges. The current in the electrical field breaks down the gas between the plates in the gap space between the electrodes. The previous study has identified gap space, gas composition, voltage, treatment time, bacteria type, and initial bacterial population as experimental characteristics that influence plasma's bactericidal efficacy (Ziuzina et al., 2013). It is critical to understand how these characteristics influence microbial inactivation. Misra et al. (2013) discovered that the dielectric is critical to the proper functioning of the discharge. The gap space is the distance between the ACP system's electrodes; it is determined by the product and package being treated, but it allows electrical currents to pass across the gaps. As a result, DBD plasma delivers consistency and stability in sample decontamination by operating across a large area (Cullen et al., 2014).

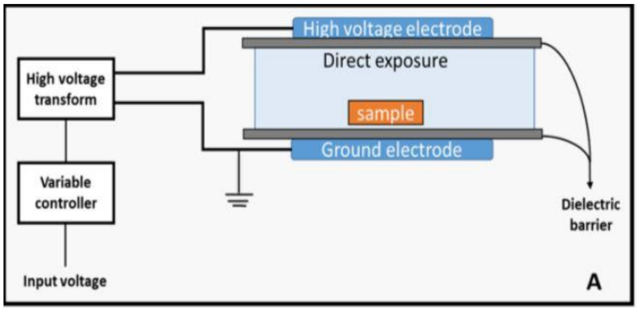


Figure 10. Schematic of experimental lab setup using dielectric barrier discharge atmospheric cold plasma system "Reprinted from [Moore, 2015]."

Atmospheric Cold Plasma Treatment System treatment technology has been shown to successfully inactivate bacteria, molds, yeasts, and other pathogens on agricultural products including fruits & vegetables, herbs and spices, food grains and nuts, and meat and meat products (T. Moiseev et al., 2014). ACP is generated by the input of electrical energy which is formed within a DBD device. The plasma is an ionized gas that consists of ions, electrons, neutral species, and UV-visible light. When ionizing gas in an ACP system, over 75 unique chemical species and 500 chemical reactions occur at four different time scales (nano-, micro-, milli-, and seconds). For air, this process generates ozone, nitrogen oxides, peroxides, and other reactive gas species (RGS). The major advantage of ACP is its ability to provide a non-thermal treatment that can reduce the presence of chemical residues, pesticides, and environmental pollutants commonly found in cereal crops.

Material and Methods

The first generation of weevils utilized in this study originated from a colony at Texas A&M University in College Station, Texas, which was maintained in the Entomology Laboratory. The PHEED Laboratory at Texas A&M University in College Station, Texas, was where the remainder of the generations were breaded. The weevils were housed in glass jars covered with porous Kimtech® Wipes and fed 75 grams of cowpea seeds (Vigna unguiculata) daily. The weevils were incubated in an 80.6 ± 1 ° F (27 ± 1° C) room temperature environment with a relative humidity of 60 ± 5% at all phases of their development. Grain for the experiment was purchased from Azure Standard, Dufur, Oregon, a company that produces natural, organic, non-GMO, and environmentally friendly products.

Treatment of Adult Insects

216 mature insects were placed in a Cryovac ® bag (SEALED AIR®, Charlotte, NC) with an inner width and length of 20 mm x 30 cm, flushed and filled with 4- 5 cm of MAP gas (65% O_2, 30% CO_2, and 5% N_2), heat-sealed, and treated in three replications. The MAP gas packages containing mature cowpea weevils were subjected to voltages of 20, 50, and 70 kV for 1, 2, and 3 minutes.

The insects remained in the packaging for 30 minutes after treatment as the gaseous species continued to move throughout the package. The adults were removed from the Cryovac ® bag and placed in glass jars covered with porous Kimtech® Wipes and fed 15 grams of cowpea seeds (Vigna unguiculata) daily. The treatments were then observed for 0-72 hours (PTRT in a room temperature environment. The mortality of the insects was recorded. If the adult insect did not move after being touched with the bristles of a paintbrush, it was declared dead.

Hypoxia Treatment of Adult Insects

To allow the insects to metabolize the oxygen contained in the bag, 216 mature insects were put in a Cryovac ® LDPE bag with an inner width and length of 20 mm x 30 cm and heat sealed. During the PTRT period, the insects were kept in the packaging for 0-72 hours, and the number of surviving cowpea weevils was counted daily. If the adult insect did not move after being touched with the bristles of a paintbrush, it was declared dead.

Electric Treatment of Adult Insects

216 adult insects were placed in a Cryovac ® bag (SEALED AIR®, Charlotte, NC) with an inner width and length of 20 mm x 30 cm heat sealed and treated. The packages containing adult cowpea weevils were exposed to voltages of 20, 50, and 70 kV for 1,2, and 3 minutes in three replications. After treatment, the package was rehomed and examined for 0-72 hours during the PTRT period. If the adult insect did not move after being touched with the bristles of a paintbrush, it was declared dead. Cowpea weevils were only exposed to the electrical component of the ACP system during these treatments, avoiding the formation of RGS that occurs when the bags are flushed and filled with MAP gas (65% O_2, 30% CO_2, and 5% N_2).

Analysis of Insects

Percentage mortality was used to assess the impact of ACP treatment on cowpea weevil viability. All of the insects were examined using an Insten Magnifying Glass and the naked eye. Adult insects were examined for IMR and PTRT mortality after treatment.

Statistical Analysis

SPSS Statistics was used to conduct the statistical analysis (version 27.0.1.0, IBM Software). The means of all ACP-treated samples and their respective untreated controls were analyzed using SPSS and compared using Fisher's least significant difference at the 0.05-confidence interval. This software also provided the standard deviation, minimum, and maximum values. The IMR of the adults was calculated as the number of treated adults who died/total number of treated alive adults per trial × 100%. The PTRT mortality of the adults was calculated as the number of treated adults who died/total number of control adults that died over a 72-hr. period per trial × 100%.

Results and Discussion

In **Figure 11** we found that 20 kV had total percent mortality of 46, 67, and 80 % for 1, 2, and 3 minutes respectively. These results show that as the treatment time increased the percent mortality also increased. Also, an immediate effect of the ACP treatment at 20kv was noted for the 2 and 3-minute treatments. By the first 24 hours, 22% of the insects had died when treated with 20kv for 2 minutes, and 32% of the insects had died when treated with 20kv for 3 minutes, compared to only 10% for 20kv at 1 minute. At 50 kV, at 1, 2, and 3 minutes, total percent mortality was 48, 61, and 91%, respectively. These findings reveal that as treatment time and voltage were increased, mortality increased. From the 20 kV treatment at 3 minutes to the 50 kV treatment at 3 minutes, there is a 10% increase in mortality within 72 hours. This treatment voltage and treatment time of 3 minutes yielded the best results.

Total percent mortality for the 70 kV treatment was 50, 60, and 86%, respectively, at 1,2,3, minute. These results back the findings from the other two treatments, which showed that as treatment duration and voltage rose, mortality increased. When the voltage remains constant, there is also a trend of increased mortality. Several experimental parameters, including the gap space between the electrodes (Yun et al. 2010; Miao and Yun 2011), gas composition (Lee et al. 2011; Kim et al. 2011; Du et al. 2012), supply voltage (Yun et al. 2010), treatment time (Miao and Yun 2011), insect type (Ziuzina et al. 2021), and direct and indirect plasma exposure (Ziuzina et al., 2021), are involved in the effectiveness of ACP treatment. The causes of insect death utilizing atmospheric cold plasma (ACP), as outlined by Ziuzina et al. (2021), have been postulated throughout the literature (Gallagher et al. 2007). There are, however, only a few studies. The supply voltage and treatment time are two factors that influence species formation; at higher voltages, reactive species are generated at a faster pace than at lower voltages (Keener et al., 2012). This also enables the production of a larger concentration of reactive species in less time.

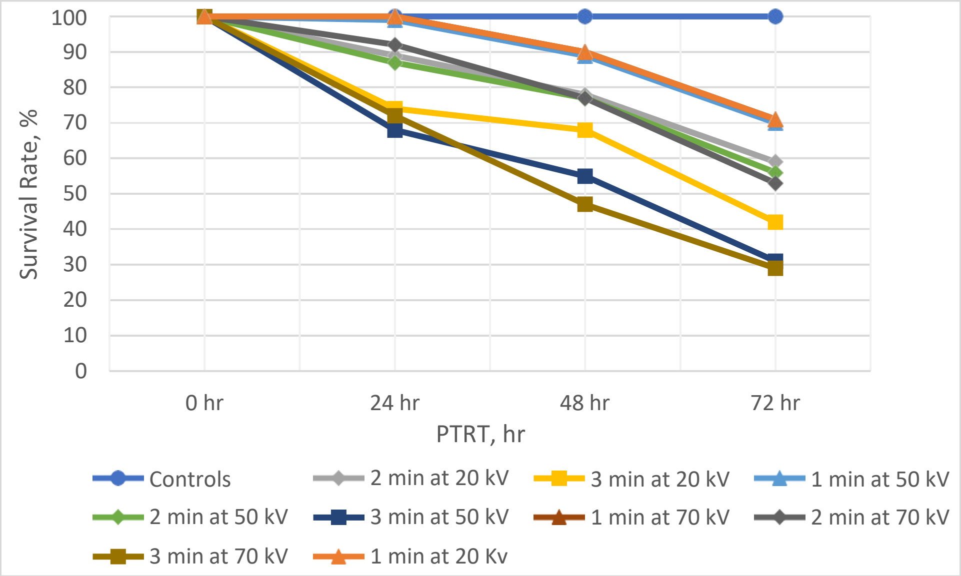


Figure 11. Treated Adults Cowpea Weevils at 20, 50, and 70 kV for 1, 2, and 3 min (PTRT - 72 hr.)

Insects in Hypoxia

Hypoxic environments are those with low levels of oxygen. When the oxygen content of the gas is lower than that of regular air, for example, when it is purposely reduced for particular uses, the resulting gas is known as hypoxic air or low oxygen air (Njoroge et al., 2019). Various studies have explored pest mortality in controlled environments, such as using high CO_2 and/or low O_2 in conjunction with temperature and relative humidity changes (Calderon et al., 1980, Donahaye et al., 1996; Mbata et al., 2000; Ofuya et al., 2002). Additional research analyzed the effect of varied CO_2 and reduced oxygen levels on *Sitophilus spp.* mortality, as well as the effect of reduced oxygen levels on *Tribolium castaneum* mortality (Emekci et al., 2002; Carli et al., 2010).

Adults and various life stages of insects have been proven to succumb completely when oxygen levels fall below 5% (Navarro, 2012; Njoroge et al., 2018). Additional study has been conducted to determine the particular processes that cause insects to die under hypoxia, such as whether death is caused by a lack of oxygen rather than an increase in carbon dioxide (Bailey, 1955), or whether death is caused by desiccation rather than suffocation (Murdock et al., 2012). While changing atmospheres have been demonstrated to kill stored-product pests in a matter of days, there is evidence that some insects may be able to withstand the impacts of reduced oxygen and increased carbon dioxide (Navarro, 2012). Insects adapt to hypoxia by expanding tracheal diameters and the number of tracheoles, lowering respiration and lowering metabolic rates (Henry et al., 2004; Zhou et al. 2007, 2008). Females may deposit eggs before becoming immobile (Yan et al., 2016), leaving behind offspring that may continue to develop and cause infestations if the hypoxic environment.

Several documented studies have shown how oxygen levels rose under airtight settings after falling below 5%, most likely as a result of undetected airtight seal breaches (Yan et al., 2017; Kharel et al., 2018). Despite the increase in oxygen, the stored grain and flour retained their quality. This is because insects died when oxygen levels dropped below 5% and the containers were sealed for at least 90 days. Insects have a diverse set of adaptations that allow them to cope with more or less severe hypoxia in a variety of aquatic and terrestrial environments (Spomer et al., 1998). Hypoxia adaptations include the capacity to convert from aerobic to anaerobic metabolic pathways (with associated end product synthesis), the ability to substantially lower basal metabolic rates, changed behaviors and expanded tracheal system sizes (Spomer et al., 1998). Many insects have evolved adaptations that allow them to spend part of their lives in hypoxia or anoxic environments. Areas with persistent hypoxia, such as high elevations and the insides of mammalian stomachs, are among these ecological conditions, as are microhabitats with transitory hypoxia, such as interior new dung and carrion, beneath the ice, in sealed containers, and momentarily submerged substrates. Each of these settings offers a distinct niche for specific insect species to thrive. The hypoxia and ACP treatments were compared to better understand the exact application of the ACP system and which components of the mechanism are lethal to pests.

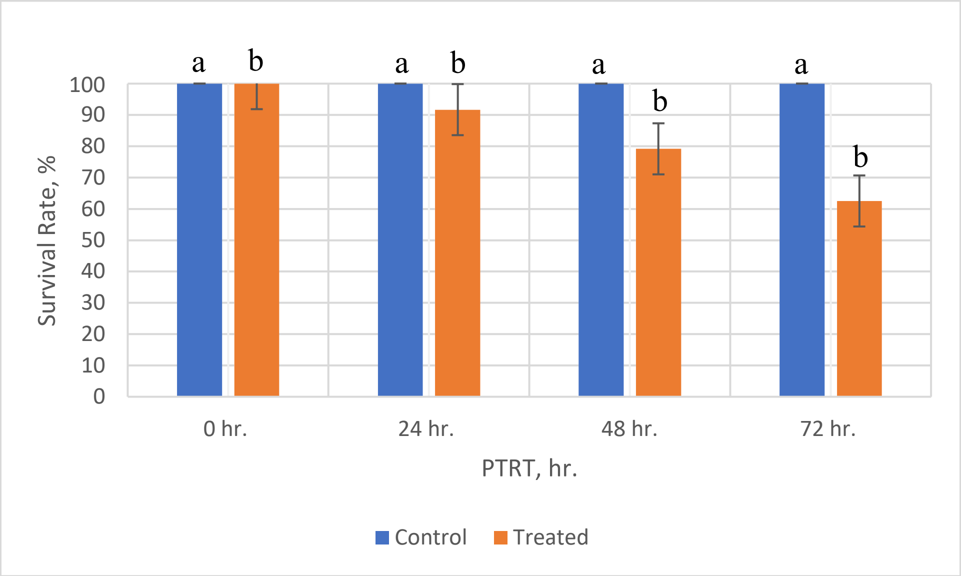


Figure 12. The survival rate of hypoxia treatment of mature cowpea weevils for a period of 0 – 72 hr.

Figure 12 shows the survival rate of cowpea weevils during periods ranging from 0 to 72 hours. Hypoxia treatments had an estimated survival rate of 100 and 91% at 0 and 24 hours, respectively, compared to the controls, who had a similar rate. After 24 hours, the survival rate begins to decline. This is due to the insects' inability to metabolize the oxygen in the bag due to a shortage of oxygen (Yan et al., 2016). According to the findings of this study, hypoxia treatment did not yield great results for cowpea weevil mortality. These results confirm previous findings that insects have a broad range of adaptations that allow them to survive with more or less severe hypoxia in a variety of aquatic and terrestrial habitats. In addition, literature shows that if severe oxygen levels fall below 5% with increased CO_2 production, then you will see the susceptibility insects have to hypoxia treatments

Effects of electricity on insects

As demand grows, so do worries about the impact of agricultural pesticides on the environment, necessitating the development of nonchemical quarantine procedures to fulfill export standards. Commodity tolerances and processing procedures have primarily influenced the sorts of physical treatments employed (Neven 2003). Temperature extremes, such as cold, are used in the most popular physical treatments (references that used cold temperature extremes as treatments). The use of controlled or modified atmospheres is a frequent physical treatment (low oxygen, elevated carbon dioxide). The use of electricity to combat infesting insects has been investigated thanks to advances in technology. Ionizing radiation, microwaves, ultraviolet radiation, infrared radiation, radiofrequency, electron beam, X-rays, and electricity are some of the treatments available (Neven 2003). An electric field is a region that surrounds an electric charge and allows it to exert a noticeable force on another electric charge. Electric fields, particularly at high voltage, cause a variety of electrostatic phenomena, some of which might be used to provide effective pest control methods (Kusakari et al., 2020).

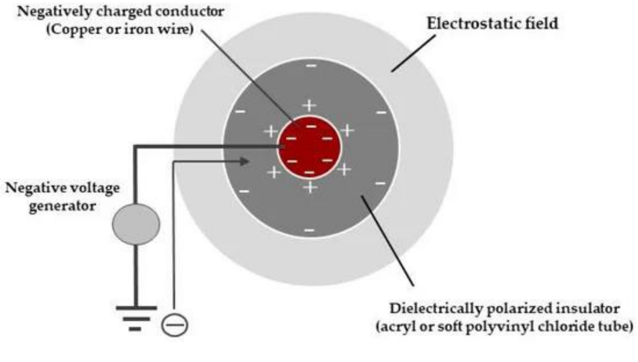
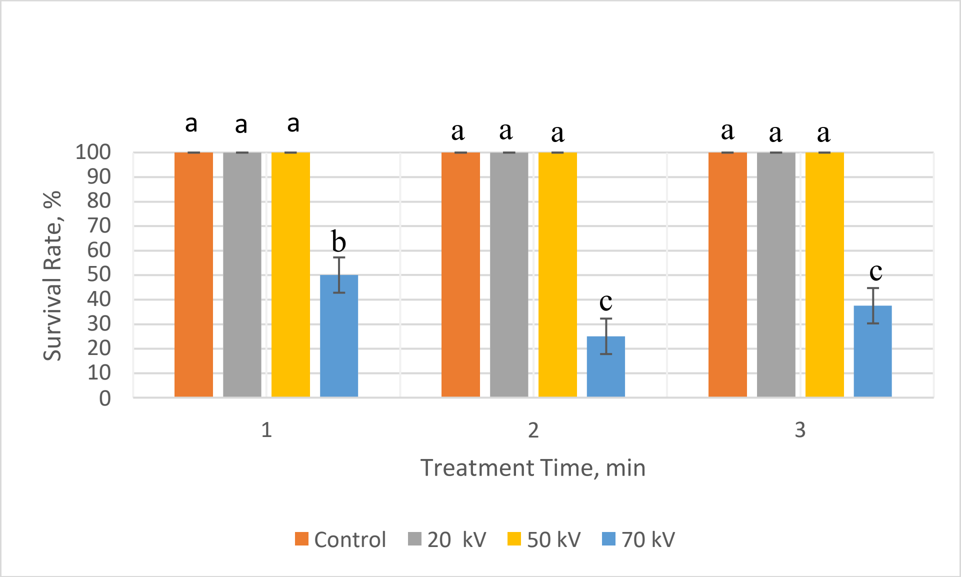
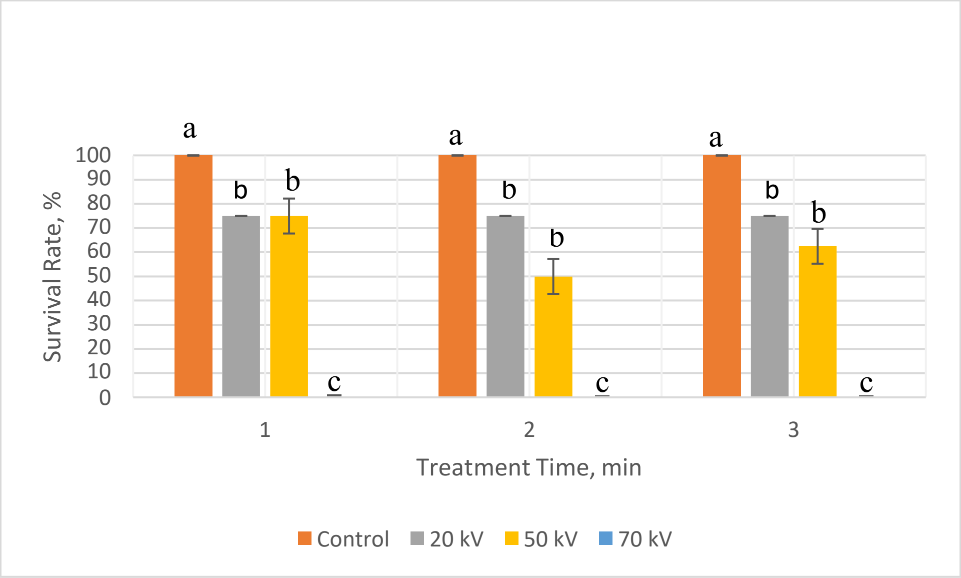


Figure 13. Schematic representation of the electrostatic field produced by a negatively charged insulator, covering a negatively charged conductor "Reprinted from [Jones et al., 2002]."

The most prominent phenomenon in electric fields was the strong attraction force created in non-discharging electric fields such as electrostatic and static electric fields. According to previous research, the earthed net depleted the negative charge concentrated on the net side of the insect cuticle layer by applying suitable voltages (Kusakari et al., 2020). These insects turned net positive and gravitated toward the insulated conductor wires' negative charge (Kusakari et al., 2020). The increase in voltage supplied to the insulated conductor wire increased the attraction force in direct proportion to the increase in voltage applied to the insulated conductor wire (Kusakari et al., 2020). During skeletal muscle motions, the attracted insects created electricity, however, this energy flowed to the earthed net rather than being used to neutralize their positive charge, which was believed to be a viable countermeasure to counteract the insulated conductor wire attraction power (Kusakari et al., 2020).



(a)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Survival Rate, %
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Treatment Time, min
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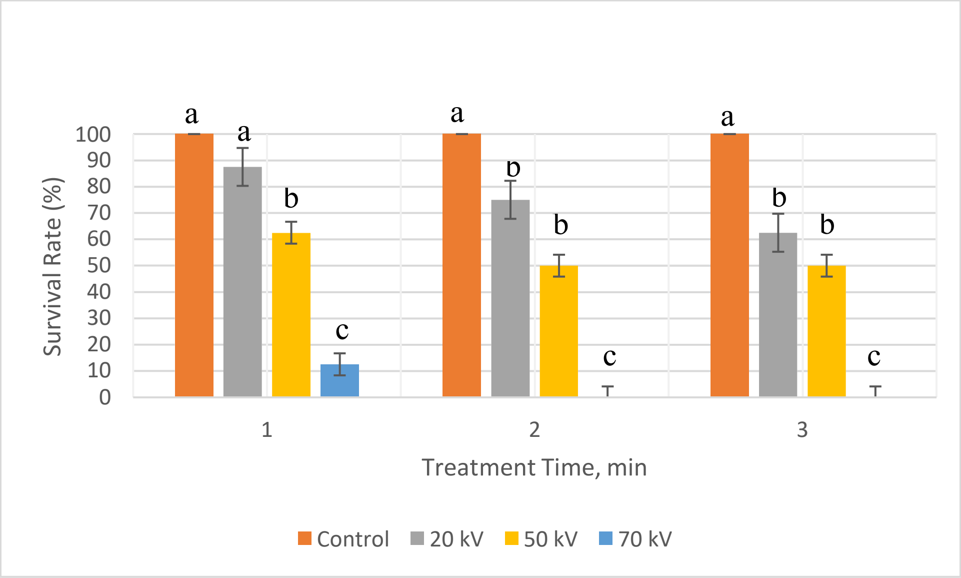
(b)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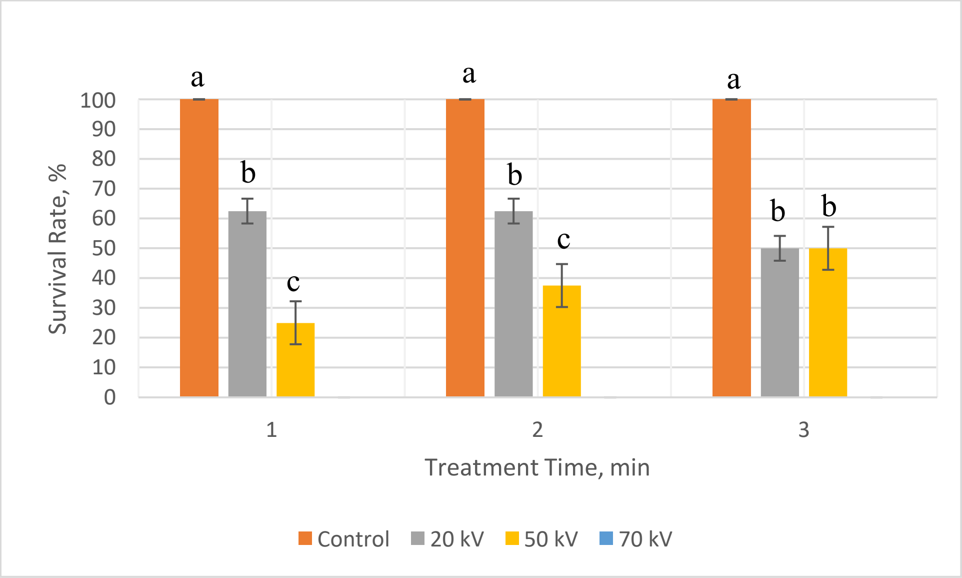
(c)



(d)

Figure 14. The survival rate of cowpea weevils, treated with electricity at 20, 50, 70 kV for 1,2, and 3 minutes and examined for PTRT for (a) 0 hr., (b) 24 hr., (c) 48 hr., and (d) 72 hr.

Figure 14 shows, as a function of treatment time, voltage levels, and post-treatment retention time (PTRT), the survival rate of the control and treated insects: (a) 0 hr. PTRT for cowpea weevil adults for 0-3 minutes at 20-70 kV, (b) 24 hr. PTRT for cowpea weevil adults for 0-3 minutes at 20-70 kV, (c) 48 hr. PTRT for cowpea weevil adults for 0-3 minutes at 20-70 kV, (d) 72 hr. PTRT for cowpea weevil adults for 0-3 minutes at 20-70 kV. Different letters represent significant differences at the level of $p < .05$. As a result, the findings give a solid explanation for an electric field screen's capacity to confine attracted flies and potentially lead to insect death. According to this study, electricity played a significant ($P < 0.05$) role in the mortality of cowpea weevil adult insects. At higher treatment voltage (50-70 kV) and increasing PTRT, cowpea weevil mortality was 100% after the 0-72 hr. PTRT.

Conclusions

Phosphine gas has been utilized as an effective fumigant for disinfesting stored grains and other commodities for more than four decades around the world. With recent constraints on the manufacturing of the only alternative, methyl bromide, its usage as a safe fumigant of stored products has become even more crucial. In several regions, widespread phosphine resistance has arisen in various species of stored-product insects, which have resulted in increased infestations (T. Moiseev et al., 2014; M. Ferreira et al., 2016). Chemical and physical treatments are typical management strategies, although they can leave hazardous chemical residues on grains, change the nutritious content of grains (especially temperature treatments), and have other drawbacks. Cold plasma can manage postharvest fungus, mycotoxins, and insect pests in stored wheat grain, according to evidence from multiple international research studies (Cullen et al., 2014; Bárdos et al., 2010; Pankaj et al., 2014; T. Moiseev et al., 2014). According to this study, ACP is an effective treatment method, because it combines not only reactive gas species but also electricity which cowpea weevils are susceptible to. However, hypoxia was not seen to significantly affect the overall mortality of cowpea weevils in this study.

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CHAPTER V

OBJECTIVE 3: ATMOSPHERIC COLD PLASMA TREATMENT EFFECTS ON COWPEAWEEVIL ADULT FERTILITY

Abstract

Legume seeds are the primary protein supply in many underdeveloped nations. However, they suffer significant losses during storage (Pérez Mendoza et al., 2004). More than 30% of output is lost between harvest and consumption, with the proportion being higher in other regions due to the extended storage period. During storage, pests such as the one studied in this research damage the grain, making it nearly difficult to sell and resulting in financial losses for farmers (Pérez Mendoza et al., 2004). *Callosobruchus maculatus* is a common pest for cowpea legume. Due to its prevalence, synthetic pesticides are used heavily to control it (Ziuzina et al., 2021). Therefore, environmental safety and public health are an increasing concern, as these insecticides hurt both. In addition, the overuse of these insecticides has led to insect resistance (Ziuzina et al., 2021). Given that adults are already more challenging to kill, based on literature, this is a serious problem (Ziuzina et al., 2021). Methyl bromide and phosphine are both toxic to a wide range of insect pests and have long been used to control beetles that damage stored goods (US EPA). Although these fumigants are effective and cost-effective, they have resulted in unintended consequences such as ozone depletion, environmental pollution, pest resistance, and unintentionally affect non-targeted species (Ziuzina et al., 2021). Due to the problems of resistance and risk to environmental safety, other treatment methods are needed. ACP is a revolutionary treatment approach that may be used to manage pest populations in storage facilities. Previous research, as well as this present study, has demonstrated the effectiveness of ACP treatment in increasing cowpea weevil mortality at three key stages of life: egg, first instar, and adult. Furthermore, the fertility of treated cowpea

weevils was impacted by ambient cold plasma when compared to controls. According to research, insect sterility is caused by sub sterilizing doses of ionizing radiation. When partially sterile males' mate with wild females, the radiation-induced negative consequences are transmitted down to theF1 generation. This study looked at whether ACP treatment reduced the reproductive rates of adult cowpea weevils, which could help with the sterile insect treatment approach. The reproductive rates of treated cowpea weevil adult insects are examined and discussed in this study. The results indicate that ACP can be used to prevent adult cowpea weevils from reproducing.

Introduction

The sterile insect technique is an environmentally-friendly insect pest control method that involves mass-rearing and sterilizing a target pest using radiation, releasing sterile males by air over defined areas where they mate with wild females, resulting in no offspring and a declining pest population (IAEA, 1998–2022). The sterile insect technique (SIT) is one of the most eco-friendly insect pest management techniques currently established. Irradiation with Gamma rays and X-rays are used to sterilize mass-reared insects, ensuring that they can no longer reproduce while remaining sexually competitive (Follett et al., 2007). Irradiation as a phytosanitary treatment for agricultural commodities is gaining popularity across the world, especially with the release of the International Plant Protection Convention (IPPC) standard, which approves and promotes trade based on this method of disinfestation (Follett et al., 2007). Irradiation is efficient against beetles in a wide range of dosages that do not degrade the quality of most goods (Follett et al., 2007). Irradiation, unlike other disinfestation treatments, does not need the pest to be killed immediately to maintain quarantine security, therefore live but sterile or nonviable insects may be included with the exported goods, making pest inspection unnecessary (Follett et al., 2007). In the United States, generic irradiation treatments for controlling a wide range of insects in all goods have been authorized. Transgenic methods involve the random integrations of a gene construct that drives the expression of a foreign gene or DNA fragment using a cell- or tissue-specific promoter segment. (Follett et al., 2007). SIT does not use transgenic methods (IAEA, 1998–2022). The benefits of SIT are, sterile insects do not self-replicate the pest's reproductive cycle, also known as autocidal control, is species-specific, and non-native species are not introduced into ecosystems by the SIT (IAEA).

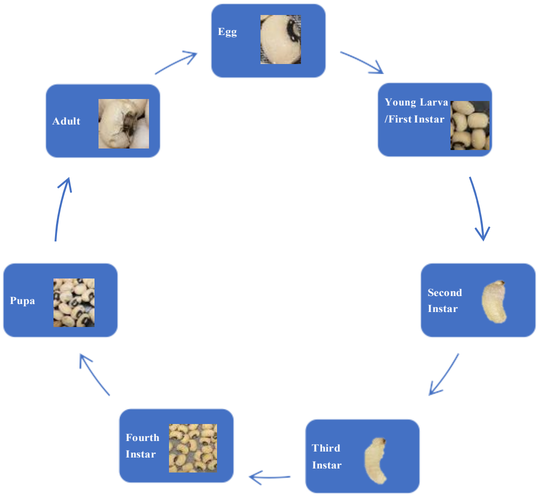

Figure 15. Cowpea Weevil Lifecycle Diagram

Existing literature on cowpea weevil fertility covers thermal stress, aromatic treatments, and irradiation with Gamma rays and X-rays. In thermal stressors, Liu et al., (2021) showed that in cowpea weevils, temperature stress hurts male fertility and sperm competitiveness. Cowpea weevil, on the other hand, has varying levels of cold resistance. At 0°C, cowpea weevil eggs, adults, larvae, and pupae are in the order of cold tolerance. In addition, cowpea weevil development phases (eggs, larvae, pupae, and adults) can be controlled at 15 °C for 3 hours. In aromatic treatments, Riffi et al., 2021 showed that the essential oil of *Artemisia herba alba Asso* has a significant impact on *Callosobruchus maculatus* brunches fecundity. The essential oil

Artemisia herba alba Asso causes a complete absence of developed eggs at a concentration of 1.24g/4.6L. Mentioned above, irradiation with Gamma rays and X-rays are used to sterilize mass-reared insects, ensuring that they can no longer reproduce while remaining sexually competitive (Follett et al., 2007). Irradiation as a phytosanitary treatment for agricultural commodities is gaining popularity across the world and is being utilized as an integrated pest management technique.

Insecticides for Infertility and Sterility

Insecticides have been used to render insects sterile in several studies (Kumar et al., 2011; Rimoldi et al., 2008; Pavela, 2013). The acute toxicity of seven naphthoquinones was evaluated against adults of the house fly (*Musca domestica*) in one study (Pavela, 2013), with plumbagin being the only one to exhibit significant acute toxicity (LD50 = 21 and 18 g for females and males, respectively). For plumbagin, the effectiveness of sublethal dosages (LD30) was investigated. *M. domestic's* lifespan, fecundity, and fertility were all reduced significantly by sublethal dosages of plumbagin (Pavela, 2013). The treated females oviposited 22.1–30.5 eggs per female on average, but the untreated females oviposited 129.3 eggs per female, which was substantially fewer than the control of 224.8 eggs per female (Pavela, 2013). When compared to the control, eggs oviposited by treated females had a much lower hatching capacity (50%) than eggs oviposited by untreated females, which had a hatching capacity of 99 percent (Pavela, 2013).

In another study, leaves of the *Parthenium hysterophorus* plant were gathered from the surrounding area and processed into a concentrated extract, which was then collected and kept at 4°C as a 1,000 ppm stock solution for treatments (Kumar et al., 2011). These solvent extracts were then used to study the fecundity and fertility of *Aedes aegypti.* When compared to controls, extracts of 1,000 ppm concentration generated from the leaves of P. hysterophorus using different solvents caused 70–100% oviposition repellency, resulting in significantly lower fecundity (Kumar et al., 2011). Diethyl ether extract, which induced 99.7% effective repellency, caused the highest and most substantial reduction in fecundity in Aedes aegypti (Kumar et al., 2011). The acetone and hexane extracts, on the other hand, had the lowest repellency (70–74%) (Kumar et al., 2011). The extracts of benzene and petroleum ether were likewise shown to have a 93–94 percent effective repellency against oviposition (Kumar et al., 2011).

After being fed an artificial diet ad libitum, one study revealed how susceptible *Chrysoperla externa* adults were (Rimoldi et al., 2008). The proportion of eggs deposited by females in the initial oviposition was almost always lower than in the 48 and 72 h oviposition (Rimoldi et al., 2008). Fertility was not altered by methoxyfenozide treatment; however, fecundity was inhibited only for the first 24 hours (Rimoldi et al., 2008). When administered to *C. externa* eggs, Spinosad did not affect fecundity or fertility (Rimoldi et al., 2008).

Sterile Insect Method

The sterile insect method was invented in the United States and has been successfully utilized for over 60 years. It is being used on six continents currently. Suppression, eradication, containment, and prevention are the four strategic approaches for using sterile insects as part of area-wide integrated pest control. Considering ACP shows promise in suppressing the fertility rate of cowpea weevils, it can be used in conjunction with the SIT approach as it is incorporated into the agricultural industry.

Materials and Methods

The study was carried out at Texas A&M University College Station campus. The weevils were incubated in an 80.6 ± 1 ° F (27 ± 1° C) room temperature environment with a relative humidity of 60 ± 5% at all phases of their development. Grain for the experiment was purchased from Azure Standard, Dufur, Oregon, a company that produces natural, organic, non-GMO, and environmentally friendly products.

Adult Treated Insects Mating

For these treatments, 216 adult cowpea weevils were exposed to 70 kV for 3 minutes and 0 hr. of PTRT. They mated in a room temperature environment for 1-2 hours. Adult females laid single fertilized eggs on the outside of a bean after being inseminated. Individual eggs (0.75mm long) are oval or spindle-shaped, transparent, glossy, and securely adhered to the surface of the bean. The eggs can easily be seen. The actively treated insects were then isolated from the eggs that had been deposited. Following separation, the number of eggs was counted and recorded using an Insten Magnifying Glass. This fertility test was replicated three times and compared to the controls.

Statistical Analysis

SPSS Statistics was used to conduct the statistical analysis (version 27.0.1.0, IBM Software). The means of all ACP-treated samples and their respective untreated controls were analyzed using SPSS and compared using Fisher's least significant difference at the 0,05-confidence interval. This software also provided the standard deviation, minimum, and maximum values.

Results and Discussion

In laboratory cultures, females produce 30 to 100 eggs over their lifetime. Because bean beetles are a pest of stored products, the laboratory setting is quite comparable to what these insects would encounter in nature (Beck et al., 2013). ACP treatment strongly affected the fecundity of mature female cowpea weevils in this study. The average number of eggs laid varied from 163 – 120 for treated insects, compared to 360 – 310 for non-treated insects (**Figure 16**). Treated cowpea weevils laid approximately 50% fewer eggs than the non-treated samples. These findings indicate that after a 70kv and 3 minutes of treatment, surviving cowpea weevils will lay fewer eggs for future generations. However, there is concern that surviving insects may have adapted to the ACP treatment conditions and/or become resistant to treatment. More research is needed.

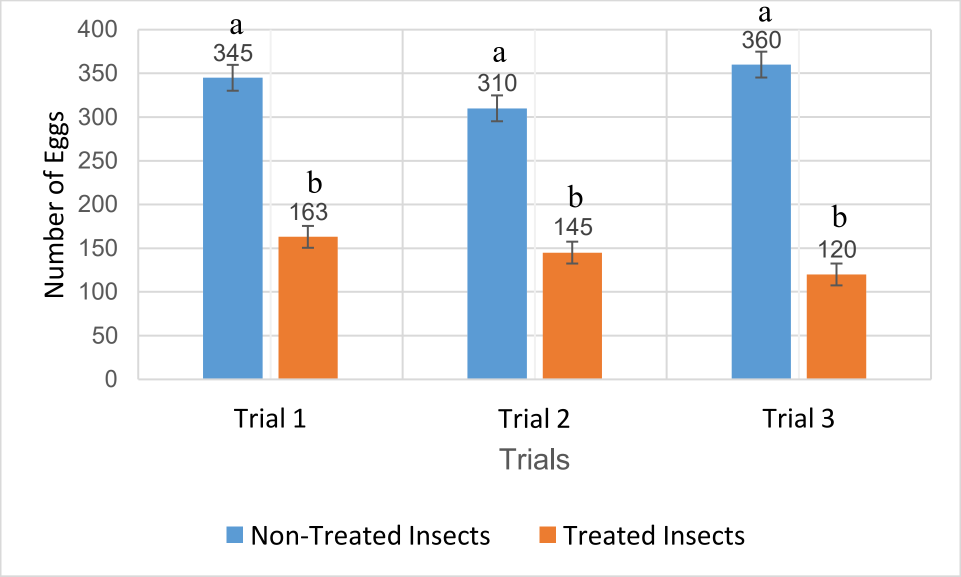

Figure 16. These tests were performed on treated insects at 70kV for 3 minutes. The controlled group laid more eggs than the treated group.

Given the above context and the results yielded, ACP treatment might have rendered the female cowpea weevil infertile. For non-treated cowpea weevils, 92% successfully emerged into adults. Although we did not observe the hatch rate of eggs from treated adults, the viability assay revealed a decrease in fertility based on visual inspection (approximately 52%).

Conclusion

Atmospheric cold plasma (ACP) has specific advantages for food decontamination. The term "plasma" refers to an electrically neutral gas made up of molecules, atoms, ions, and free electrons (Selcuk et al., 2008; Schmidt et al., 2019). This revolutionary treatment technology has potential applications in integrated pest management. According to this study, ACP successfully suppressed the reproductive rates of adult cowpea weevils when compared to controls. This indicates that if ACP treatment does not effectively kill all cowpea weevils during the three critical life phases, it can prevent the following generation from developing. Future work will involve observing hatch rate and genetic traits that may have allowed the adults to survive the initial treatment. This will give us more information as to if there is a biological reason correlating to resistance to ACP treatments, or if the ACP treatment played a role in mortality and fertility.

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